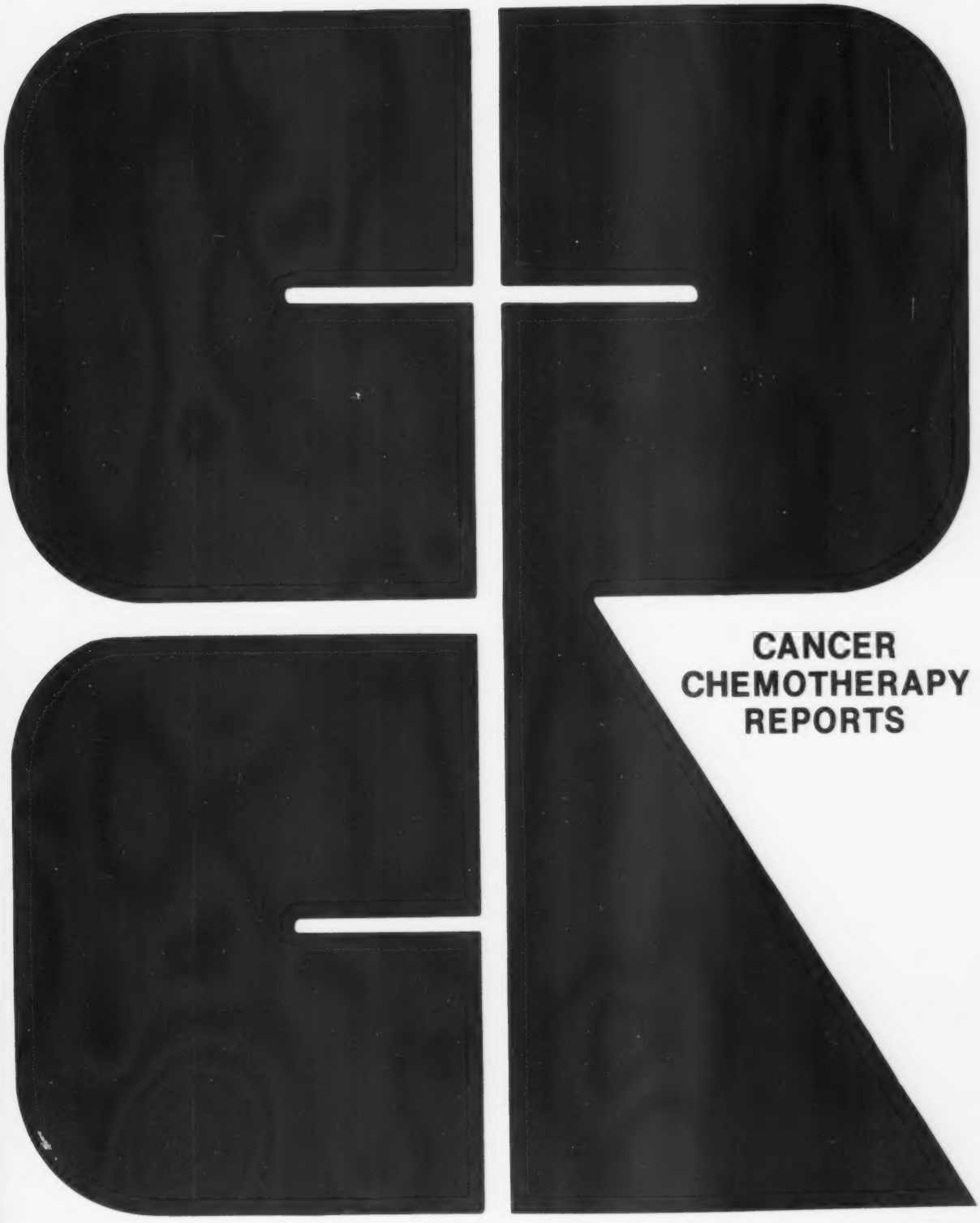

CANCER CHEMOTHERAPY REPORTS

Part 3

July 1975
Vol. 6, No. 1

Cancer Chemotherapy Reports

National Cancer Institute

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Division of Cancer Treatment

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Part 3 is issued by Drug Research and Development, Division of Cancer Treatment, National Cancer Institute

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High-Dose Methotrexate Therapy Meeting

Division of Cancer Treatment

National Cancer Institute

Bruce A. Chabner and Milan Slavik, editors

Bethesda, Maryland
December 19, 1974

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The Secretary of Health, Education, and Welfare has determined that the publication of this periodical is necessary in the transaction of the public business required by law of this Department. Use of funds for printing this periodical has been approved by the Director of the Office of Management and Budget through November 30, 1976.

Introduction: Perspectives on High-Dose Methotrexate (NSC-740) Therapy[1]

Bruce A. Chabner[2] and Milan Slavik[3,4]

Recent reports of the successful use of high-dose methotrexate (MTX) with citrovorum factor (CF)[5] rescue in the adjuvant treatment of osteogenic sarcoma (1), as well as in other clinical situations, have greatly expanded the potential of this agent and prompted further clinical investigations. In this context important questions have been raised as to the rational and safe use of this new form of therapy, since it is clear from the experience of several groups that serious and often unpredictable myelosuppression may occur with high-dose MTX (2). Recently, 12 drug-related deaths have been reported to the Investigational Drug Branch, National Cancer Institute, by experienced investigators from several major cancer centers. Therefore, it is appropriate that the studies in this issue be presented at this time in an attempt to provide perspective and to furnish guidelines for further clinical work. The therapeutic indications for the use of high-dose MTX, here defined as doses in excess of 1 g/m^2 or 20 mg/kg, include at present only adjuvant treatment of osteogenic sarcoma and treatment of metastatic osteogenic sarcoma. In both situations, while the ultimate value of the treatment has not been established, therapeutic benefit, in terms of a delay in recurrence following surgery or a partial response of metastatic disease, has been documented in a setting where there are few other alternatives. Other work indicates potential effectiveness in this form of therapy for refractory acute leukemia (3), bronchogenic carcinoma (4), and head and neck cancer (5), although these results need verification through more extensive trial. In all other clinical trials it appears reasonable at present to use high-dose MTX only after clear failure of the safer moderate dose regimens (100-1000 mg/m^2) or in a concurrent randomized trial with a lower dose regimen; this type of trial is necessary in order to establish whether the added risk and considerable cost of high-dose MTX are justified by superior therapeutic results. Careful patient selection is necessary in the use of high-dose MTX. The importance of rapid drug excretion in limiting toxicity, as indicated by the pharmacokinetic studies in this issue, would preclude the inclusion of any patient with renal impairment or a third-space reservoir (6,7) such as ascites or large pleural effusion.

The pharmacokinetic work presented in this issue provides a background for the rational planning of therapy and a better understanding of the mechanisms of toxicity. After doses of 50-200 mg/kg, peak levels of MTX in the blood ranged from 0.1 to 1.0 mM and were roughly proportional to dose. A rapid decline in plasma concentration occurred in the period after infusion, with an initial half-life of 120-157 minutes in nontoxic patients (8,9). A prolonged final phase of excretion was seen by Stoller et al (9) at late time-points, but appeared to be effectively neutralized by CF and was of no toxicologic consequence in patients excreting the drug normally. The prompt urinary excretion of MTX appeared to be vital to the avoidance of toxicity; small changes in renal function following MTX infusion were associated with delayed excretion, the failure of CF rescue, and severe myelosuppression in patients described by Tattersall et al (7) and Stoller et al (9). Careful monitoring of renal function and serum MTX levels provided helpful information regarding impending toxicity and the need for extended CF administration.

Routine pharmacokinetic monitoring of patients receiving high-dose MTX is clearly indicated, but the assay of MTX by the classic dihydrofolate reductase method requires biochemical expertise and special materials such as dihydrofolate. Several new assay methods have been developed and offer significant advantages in terms of simplicity and ease of performing multiple determinations. The radioimmunoassay described by Levine et al (10) requires a special anti-MTX antibody and overnight incubation. A new competitive binding assay employing *Lactobacillus casei* dihydrofolate reductase (commercially available from New England Enzyme Center, Boston, Mass) has the significant advantage of requiring only 1-2 hours to perform multiple determinations, and is probably the easiest method available for routine use.[6]

The mechanism of renal injury due to MTX is unclear from these studies. However, renal precipitation of MTX in acidic urine was shown to occur (9) and provides a strong argument for urine alkalinization and increased urine volume. A second

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[1]Methotrexate: CAS reg. No. 59-05-2; glutamic acid, *N*-[*p*-[[(2,4-diamino-6-pteridinyl)methyl]methylamino]benzoyl]-; MTX.

[2]Biochemical Pharmacology Section, Laboratory of Chemical Pharmacology, Experimental Therapeutics (EX), Division of Cancer Treatment (DCT), National Cancer Institute (NCI), Bethesda, Md.

[3]Investigational Drug Branch, Cancer Therapy Evaluation, DCT, NCI, Bethesda, Md.

[4]*Reprint requests to*: Bruce A. Chabner, MD, Biochemical Pharmacology Section, EX, DCT, NCI, Bldg 10, Rm 6N119, National Institutes of Health, Bethesda, Md 20014.

[5]Citrovorum factor: NSC-3590; CAS reg. No. 6035-86-5; glutamic acid, *N*-[*p*-[[(2-amino-5-formyl-5,6,7,8-tetrahydro-4-hydroxy-6-pteridinyl)methyl]amino]benzoyl]-, calcium salt; leucovorin.

[6]Myers CS, Lippman M, and Chabner BA. A competitive protein binding assay for methotrexate. Proc Natl Acad Sci USA. In press, 1975.

factor in avoiding MTX precipitation is the limitation of total dose and the avoidance of bolus administration (7).

In addition to defining the mechanism of renal injury due to high-dose MTX, other important areas for pharmacologic investigation include the nature of MTX transport at high extracellular concentrations, the mechanism of cytotoxicity of MTX at low and high levels, and the competitive aspects of MTX/CF interaction. Largely due to the impetus provided by the clinical results reported by Djerassi, Jaffe, and their coworkers, these studies will likely be pursued with determination in the months ahead.

In summary, the high-dose MTX/CF rescue regimen represents a promising therapeutic approach particularly in the adjuvant treatment of osteogenic sarcoma. Because of its potential toxicity and the growing number of reports of drug-related deaths, its value should be further examined only in centers where all the necessary resources and expertise are available to reduce the potential risk.

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High-Dose Methotrexate (NSC-740) and Citrovorum Factor (NSC-3590) Rescue: Background and Rationale[1]

Isaac Djerassi[2,3]

A review of the background, rationale, and our published information on the use of high-dose methotrexate (MTX) pulse infusions followed by citrovorum factor (CF) rescue may help us define better the immediate usefulness of this therapeutic modality as distinguished from its potential. The treatment method consists of two separate components which we studied in sequence. First, we tried to determine the effective and practical schedules for the administration of high-dose pulse MTX (1,2). A new approach to CF rescue was subsequently developed which aimed at protecting normal sensitive tissues without canceling all antitumor effectiveness (2-5).

In 1963, we became interested in the mechanism of resistance to folic acid antagonists. At that time it was proposed that: (*a*) MTX combines stoichiometrically (6) and irreversibly with dihydrofolic acid reductase, (*b*) that increased synthesis (7) of this enzyme is found in cells which have developed resistance to this class of agents, and (*c*) that decreased permeability of the cell to the antifol may contribute to its resistance. We felt that should these statements be correct they contained in themselves a potential approach to overcoming the resistance to antifols. If resistance was due to an excess (absolute or relative) of the enzyme (dihydrofolic acid reductase), then by introducing more MTX molecules into the cell, until the last enzyme molecule was properly bound "stoichiometrically and irreversibly," we could perhaps still destroy the so-called resistant cell. However, how could we introduce at will more molecules into cells?

In the late 1950s we studied the saturation of platelets with 5-hydroxytryptamine (Serotonin). We were able to increase 100 times the amount of intraplatelet 5-hydroxytryptamine, exceeding five times the capacity of the apparent active transport mechanism by incubating human platelets in high concentrations of the chemical. The intraplatelet concentration of 5-hydroxytryptamine was proportional to its concentration in the medium and to the time of incubation. Passive transport of 5-hydroxytryptamine following the principles of diffusion across semipermeable membranes and bypassing the physiologic active transport mechanism was apparently responsible for this observation. Although the clinical effectiveness of platelet transfusions was not enhanced as a result of this study, it nevertheless became the basis for our studies of the high-dose pulse MTX chemotherapy method.

Saturation of resistant leukemic cells with MTX was attempted clinically with an 18-hour infusion of the drug (1). We used 12 mg/m^2 of body surface area as a priming infusion to create what appeared to be an effective blood-body fluid concentration. Instead of allowing this concentration to drop, as we knew it would in 4-6 hours, we infused an additional 12 mg/m^2 of MTX every 6 hours for 18 hours at a constant rate, thus administering a daily dose of 48 mg/m^2. The effective peak level of MTX was thus maintained for a total of 24 hours. We were incubating in vivo the cells in an effective MTX concentration, extending the time of incubation from 6 hours, which would be the effect of a single dose, to 24 hours. Repeating the same infusion on the next day would extend the incubation to 48 hours and so on. We also explored priming doses of 24 and 36 mg/m^2 with appropriate maintenance infusions.

Such infusions of MTX given to ten children with acute leukemia, who had previously shown resistance to either daily oral or twice weekly intramuscular (im) MTX, induced hematologic remission in all. We were able to overcome resistance to MTX, support our working hypothesis, and propose that the term *tolerance* be used instead of *resistance* in describing lack of response to antifols (1).

The toxicity encountered in this study suggested strongly that the duration of infusion of MTX correlated better with the occurrence of side effects than the actual amount of MTX given. We explored, therefore, the efficacy and side effects of infusions designed to provide a shorter incubation time but also to maintain the product $C \times t$ (concentration × time) constant by increasing the concentration component. Infusions of 5-15 mg/kg of MTX, lasting 4 hours only, induced remissions in MTX-resistant patients with considerably fewer side effects (2). Further experience with the chronic toxicity of these infusions indicated that an interval of 3-4 weeks between MTX infusions is needed for the recovery of all normal sensitive tissues. The MTX pulses were thus devised.

High-dose MTX pulse therapy was tolerated best by patients in remission. We therefore started a

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[1]Methotrexate: CAS reg. No. 59-05-2; glutamic acid, *N*-[*p*-[[(2,4-diamino-6-pteridinyl)methyl]methylamino]benzoyl]-.

Citrovorum factor: CAS reg. No. 6035-86-5; glutamic acid, *N*-[*p*-[[(2-amino-5-formyl-5,6,7,8-tetrahydro-4-hydroxy-6-pteridinyl)-methyl]amino]benzoyl]-, calcium salt.

[2]Mercy Catholic Medical Center, Philadelphia, Pa.

[3]*Reprint requests to:* Isaac Djerassi, MD, Mercy Catholic Medical Center, Philadelphia, Pa 19143.

study of MTX use for remission maintenance in acute leukemia (6). In the course of this study, patients relapsing while receiving pulses of 5 mg/kg of MTX were reinduced with and then maintained for another even longer period of time on 10 or 15 mg/kg, always given in 4-hour infusions (2,8). Indeed, resistance was relative and further increase of the drug concentration in the extracellular fluids could apparently ensure complete saturation of all intracellular enzyme with the folic acid analog.

The very large amounts of drug required per infusion in some of the resistant patients suggested that decreased permeability of the cell to the drug may be paramount in the development of MTX-resistant cell clones (2). Leukemic cells surviving individual doses of MTX could well be selected on the basis of lesser permeability to MTX, thus creating new generations of less permeable cells. Long-term effectiveness of MTX could, therefore, be achieved only if one could use concentrations of drug allowing it to penetrate even maximally selected cells. With this in mind, we tested other dose schedules of MTX, always with a view toward using the highest possible dose consistent with safe and long-term administration.

Early in the above studies we noticed an elevation of liver enzymes 4-5 days after treatment with high-dose pulse MTX (2). In fact, this side effect precluded our initial plans to use weekly infusions (1). It was of interest that this liver damage became evident after most of the leukemic cells, as thought at the time, had already been affected (a doubling time of 3-4 days). We saw no reason, therefore, why CF given late after the MTX could not protect the liver cells without significantly protecting the leukemia cells, if lack of CF was responsible for their damage. This notion was in contrast with most published opinions at that time, with the notable exception of the work of Goldin et al (9), that CF must be given either just prior to or at least simultaneously with the administration of the antifol in order to afford protection from its side effects (10, 11). Another dogma, still perpetuated today in the instructions accompanying commercially available CF (calcium leukovorin, Lederle Laboratories), was that CF must be given in doses equimolar to those of the MTX used. Since CF does not interact chemically or otherwise with MTX and works only as a coenzyme, we saw no reason for supplying more than the physiologic amount required. We therefore explored the use of short courses of small divided doses of CF given *after* the administration of the high pulses of MTX (≥15 mg/kg). Indeed, the elevation of the liver enzymes expected 4-5 days after MTX was prevented or improved without complete cancellation of the antileukemic effect. This was the beginning of the rescue component of the MTX/CF method (2).

I should mention and explain the difference between our approach and that of Sullivan et al (12), who used MTX infusions and CF rescue well before we started our studies. They used continuous infusions of small amounts of MTX (no pulse scheduling) for the purpose of having the drug in the body fluids at all times, presumably to be available at the time of DNA synthesis. Their total dose was 30 mg/day of MTX per adult patient given in many consecutive days. Although this dose is relatively small, it would have been extremely toxic without the CF given simultaneously with the drug every 6 hours. In other words, they used a low dose of MTX in continuous infusions (no pulses) with simultaneous CF administration. This technique is ineffective in resistant and nonresistant leukemia. Our method, based on a different rationale, used high doses of MTX given in short pulse infusions with long intervals between treatments for recovery of normal tissues, and administration of CF after the antifol for the rescue of cells with cell-division kinetics different from those of the malignant cells.

The validity of our working hypothesis, namely that saturation of the malignant cells with antifol may elicit an effect where none is otherwise obtainable, was tested further in malignant tumors known to respond poorly to folic acid antagonists. The work on non-Hodgkin's lymphoma (lymphosarcoma and reticulum cell sarcoma) was exceedingly informative in this respect (3). The high-dose pulse MTX approach consistently induced remissions, although an elaborate system of gradually increasing dosage, occasionally followed by CF rescue, had to be followed on an individual basis with each patient in order to avoid overwhelming toxicity.

The experience with non-Hodgkin's lymphoma raised for the first time the possibility that the toxicity of an individual infusion of MTX may be enhanced, sometimes to a fatal outcome, by a recirculation of the drug released from tumor cells upon rapid destruction. Our experience with other childhood tumors (neuroblastoma, hepatoma, and glioma) further demonstrated that the high-dose pulse MTX approach should be adapted and adjusted to the requirements of specific tissues and cells for optimal antitumor effectiveness.

In 1969 we applied the principles derived from the leukemia-lymphoma work in children to solid tumors in adults. It soon became apparent that the antifol concentrations achievable with the dose schedules of MTX of proven value in lymphoma-leukemia were apparently inadequate for these solid tumors. Concentrations of MTX incompatible with safety were needed to affect (penetrate?) the cells of melanomas or primary tumors of the lung or breast. CF rescue was essential if this approach was to be pursued further. An intensive phase I study, based on careful trials of gradually intensified MTX pulses combined with a variety of CF rescue schedules, was carried out in patients with inoperable lung carcinoma (4, 5). A specific dose schedule of MTX/CF was eventually derived from

the accumulated experience and specific observations (5). A 300-mg/kg dose of MTX (or 18 g for a 60-kg adult) was adopted after showing its effectiveness in tumors which did not respond consistently to 100 or even 200 mg/kg. Since the rapid course of lung carcinoma precludes a trial-and-error approach to the dose requirement on an individual basis, as is feasible in less rapidly progressing malignancies, a continuous stepwise increase toward and stabilization at the 300-mg/kg level was needed to reach drug levels beyond the natural or emerging resistance to MTX of the lung carcinoma cells.

The 6-hour duration of MTX infusion for lung carcinoma was selected instead of the 4-hour duration previously used in leukemia with a view to improving the perfusion of the solid tumor masses with MTX. Longer infusions maintaining the same drug concentration would require doubling or tripling the total dose (to 900 mg/kg), and this did not seem justified in view of the consistent positive clinical responses with the smaller dose. Spreading the 300-mg/kg dose over longer periods of time (24 hours for example), while offering better opportunity for perfusion of solid masses, would reduce the drug gradient instrumental for the passive transfer.

The dose schedule of the CF in lung carcinoma was derived by close observations of tumor and host responses. The rationale for increasing the amount of CF with the increasing doses of MTX was the greater risk throughout the 6-hour interval between injections and the need to have at least some CF in circulation at this time. The im administration of CF was preferred to the intravenous route because of the rapid disappearance of CF from circulation and the flattened blood level curve one could expect from the im injection.

During the decade we have studied high-dose MTX pulses, our group has periodically investigated blood, urine, and spinal fluid levels of the drug, using the enzyme method of Werkheiser et al (13). Our observations were consistent with the considerations I mentioned above. Indeed, most of the MTX leaves the circulation in less than 6 hours when doses of up to 15 mg/kg are rapidly infused. A typical administration of 300 mg/kg of MTX, however, may result in up to 1-5 μg of MTX per ml of serum circulating 24 hours after the infusion. The most important finding, however, was that measurable amounts of MTX (1 μg/ml) could be detected in the spinal fluid of patients given 100 mg/kg of MTX. Higher concentrations (4-7 μg) were detected in patients with brain tumors given 300 mg/kg.

The rationale for the short interval (2 hours) between MTX and CF in the lung carcinoma schedule (5) was arrived at deductively. CF given simultaneously with MTX has been reported to compete with the uptake of MTX by the cell (14). This was an additional reason for administering it after the drug. Considering the lack of response of lung carcinoma to smaller doses and the fact that 2 hours after the infusion the levels of circulating MTX drop to values we have previously found ineffective, we estimated that little or nothing would be gained by further delay of the CF rescue. On the other hand, the failure of CF rescue is definitely related to the interval between MTX and CF. Since a prolonged CF rescue (3 days) was needed, as proven in patients, and 3-day protection would be given to the tumor anyway, we felt that starting CF at a time (2 hours) when the MTX levels were already suboptimal for the tumor but still very toxic to normal tissues was worth trying. The success of this approach consolidated its use. Exceptions to this rule are made in cases where there is very rapid tumor growth and inadequate response following maximal MTX doses.

The lung carcinoma dose schedule of MTX/CF rescue was applied, without modifications, to the management of osteogenic sarcoma (15, 16). We did not have the opportunity at that time to carry out a phase I study aimed at developing a dose schedule optimal for this tumor. Nevertheless, the lung carcinoma dose schedule of MTX/CF showed activity in 40% of the patients with this malignancy (15). The first and apparently encouraging adjuvant use of this treatment after surgery in osteogenic sarcoma was recently reported (17).

I would like to address myself now to the matter of toxicity associated with high-dose MTX pulses. A number of toxic manifestations previously unknown for MTX plagued us from the beginning. The side effects we reported included: (*a*) extensive mucosal ulcerations (1,2,5,8); (*b*) profound myelosuppression (1,2,5); (*c*) skin rashes and especially erythematous whole-body rash (1,2); (*d*) profound liver toxicity, acute and chronic (1,2,8); and (*e*) renal damage, acute and chronic (1,5).

The success or failure of the MTX/CF approach depends on the successful prevention and treatment of these complications. We felt, therefore, that this treatment method was a "sui generis" therapeutic "modality" rather than one more form of chemotherapy. The complexity in using it efficaciously and safely and the special facilities and experience required (1,2,8) illustrate this contention. Not the least of the facilities needed is an unlimited supply of platelet (1,2,8) and granulocyte (2,8) concentrates and a special team of clinicians (2) trained in the use of this approach. In 1966 we stated (2) "Their application for routine management of patients . . . is further complicated by the need for careful supportive therapy including platelet and white cell transfusions. It is very possible that the prolonged responses of our patients are to a large extent due to the unusual amount of time devoted to their management. Early detection and prevention of complications (infection, hemorrhage) required continued close contact with the patients and availability of the investigators on a 7-days-a-week basis for in- and out-patient care. The latter is illustrated by the need for this

clinic to function with full staff on weekends and holidays throughout the duration of the study."

High-dose MTX/CF rescue is an unreliable ally. One can use it with impunity and elation until, for often no apparent reason, it misfires. Unfortunately, the more ill the patient and the larger the tumor, the more likely this is to happen. While we may be starting to understand at least some of the factors involved in the occurrence of catastrophic toxicity, there is little we can do at the moment except withdraw from treatment before such toxicity occurs.

The toxicity of even "modest" dose schedules, such as those we developed for leukemia-lymphoma, is greater when the treatment is used for remission induction than for remission maintenance. An MTX dose which causes barely survivable toxicity during remission induction is usually taken without difficulty later in the course of remission maintenance. For this reason, perhaps the greatest potential of MTX pulses may well be in their use as adjuvants to surgery and radiation in potentially curable patients.

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Overview of Protocols on Clinical Studies of High-Dose Methotrexate (NSC-740) With Citrovorum Factor (NSC-3590) Rescue[1]

John S. Penta[2,3]

The DCT, NCI, maintains an active investigational new drug (IND) notice with the Food and Drug Administration for both methotrexate (MTX) and citrovorum factor (CF).

The IND notice for each of these drugs contains a large number of protocols utilizing "high-dose" MTX with CF rescue in a variety of solid tumors and hematologic malignancies. Ten of these clinical protocols, several of which represent the original work with MTX-CF rescue, are discussed in this paper. These protocols were obtained directly from the IND files and do not reflect changes and amendments which have not been submitted to the NCI.

In this overview, special emphasis has been given to the MTX-CF rescue in order to focus upon such variables as:

a. the dose of MTX;
b. the time over which MTX is administered;
c. the interval between injections of MTX;
d. the delay time for CF rescue;
e. the CF rescue dose and schedule;
f. the route of administration for CF;
g. the total quantity of CF administered in proportion to the total quantity of MTX administered; and
h. the meaning of "high-dose" as applied to "high-dose MTX."

The clinical studies presented here are shown in table 1. Figure 1 represents the schema and synopsis of the protocol used in study No. 2 by Jaffe and Frei. This protocol is an adaptation of that of Djerassi (study No. 1). In the study by Jaffe and Frei, vincristine[4] is administered 1/2 hour before each MTX infusion. For prophylaxis, a 2-year period of treatment is projected. Figure 2 represents the schema and synopsis of the protocol for study No. 3 by Bertino in which patients with epidermoid tumors receive either MTX alone or MTX plus CF rescue. In the rescue area of this protocol, the initial dose of MTX is 240 mg/m^2. Figure 3 shows the schema and synopsis of the protocol used by Pratt in study No. 4. It is seen that the MTX dose is fixed and not escalated to some higher value than the initial one. Other chemotherapy included adriamycin given for 2 days followed by a single pulse of cyclophosphamide.[5] This schedule of drugs is repeated monthly for 10 months.

The protocol schema and synopsis of study No. 5 (Wilbur) are shown in figure 4. In this study, the MTX dose is escalated from 100 mg/kg up to 300 mg/kg which is then followed by vincristine plus adriamycin and then vincristine plus cyclophosphamide. When MTX plus CF rescue is resumed, it is at the 300-mg/kg level of MTX. Rotation through this series of drugs is continued for 1 year. Study No. 6 (Ragab) employs MTX plus CF rescue before and after radiotherapy. This is shown in figure 5. The MTX dose is escalated from 75 mg/kg up to 150 mg/kg and then radiotherapy is started. Upon completion of radiotherapy, MTX plus CF rescue is resumed with MTX at a dose of 250 mg/kg. Also, vincristine is given 24 hours before each MTX infusion. Study No. 7 (Rosen) employs cyclophosphamide and adriamycin courses after MTX plus CF rescue; vincristine precedes the administration of MTX. The cycle of drug administration takes place over 56 days and is repeated seven times within 1 year. A brief protocol schema is shown in figure 6.

Compadri-II[6] (study No. 8) is outlined in figure 7. In this study, MTX plus CF rescue is employed in a sequence with four other drugs: vincristine, adriamycin, melphalan,[7] and cyclophosphamide. The first dose of MTX is 75 mg/kg, administered on Day 23; the second dose of MTX is 100 mg/kg, administered on Day 71; and the third and final dose of MTX is 150 mg/kg, given on Day 99 of therapy. The entire sequence of drug administration takes place over a 50-week period. Study No. 9 (Douglass) incorporates MTX-CF rescue with radiotherapy and/or surgery. The administration of MTX-CF rescue to patients with primary and metastatic disease is shown in figure 8. When MTX-CF rescue is given, the initial dose of MTX is 15 mg/kg and may be escalated up to 600 mg/kg as noted in the protocol synopsis (fig 8). Figure 9 shows the protocol schema of study No. 10 (Gilchrist). Patients with metastatic osteogenic sarcoma are stratified and then randomized to receive

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[1]Methotrexate: CAS reg. No. 59-05-2; glutamic acid, *N*-[*p*-[[(2,4-diamino-6-pteridinyl)methyl]methylamino]benzoyl]-.

Citrovorum factor: CAS reg. No. 6035-86-5; glutamic acid, *N*-[*p*-[[(2-amino-5-formyl-5, 6, 7, 8-tetrahydro-4-hydroxy-6-pteridinyl)-methyl]amino]benzoyl]-, calcium salt; calcium leucovorin.

[2]Drug Liaison and Distribution Section, Investigational Drug Branch, Cancer Therapy Evaluation (CTE), Division of Cancer Treatment (DCT), National Cancer Institute (NCI), Bethesda, Md.

[3]*Reprint requests to:* Dr. John S. Penta, Head, Drug Liaison and Distribution Section CTE, DCT, NCI, Bldg 37, Rm 6E28, Bethesda, Md 20014.

[4]Vincristine: NSC-67574; CAS reg. No. 2068-78-2; leurocristine, sulfate (1:1); VCR.

[5]Adriamycin: NSC-123127; CAS reg. No. 23214-92-8; Adria.

Cyclophosphamide: NSC-26271; CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroeth- l)amino]tetrahydro-, 2-oxide, monohydrate; Cytoxan; CYT.

[6]Compadri-II = cyclophosphamide, vincristine (Oncovin), melphalan (L-phenylalanine mustard), adriamycin, and high-dose MTX.

[7]Melphalan: NSC-8806; CAS reg. No. 3223-07-2; alanine, 3-[*p*-[bis(2-chloroethyl)amino]phenyl]-, monohydrochloride, L; L-PAM.

TABLE 1.—Ten clinical studies with high-dose MTX-CF rescue

Study No.	Description of study	Investigator	Location
1	MTX-CF in patients with lung cancer	I. Djerassi	Mercy Catholic Medical Center, Philadelphia, Pa
2	High-dose MTX in solid tumors	N. Jaffe	Sidney Farber Cancer Center, Boston, Mass
3	MTX vs MTX-CF in epidermoid tumors	J. Bertino	Yale University, New Haven, Conn
4	Protocol for prevention of osteosarcoma metastases using combination chemotherapy (adriamycin-cyclophosphamide-MTX): phase I-II study	C. Pratt	St. Jude Children's Hospital, Memphis, Tenn
5	Treatment protocol for osteogenic sarcoma	J. Wilbur	Children's Hospital at Stanford, Palo Alto, Calif
6	Phase I-II ancillary study—massive-dose MTX with CF rescue and radiotherapy in children with metastatic (pulmonary) osteogenic sarcoma	A. Ragab	M.D. Anderson Hospital and Tumor Institute, Houston, Tex
7	Vincristine, high-dose MTX with CF rescue, adriamycin, and cyclophosphamide for the treatment of resistant sarcoma in children and adolescents	G. Rosen	Memorial Sloan-Kettering Institute, New York City, NY
8	Compadri-II study	W. Sutow	M.D. Anderson Hospital and Tumor Institute, Houston, Tex
9	Management of patients with osteogenic sarcoma	H. Douglass, Jr.	Roswell Park Memorial Institute, Buffalo, NY
10	Pilot study for MTX therapy for metastatic osteogenic sarcoma—comparison of different dose levels with or without CF rescue	G. Gilchrist	Mayo Clinic and Mayo Foundation, Rochester, Minn

one of three MTX dose levels. No CF rescue is given to patients who received MTX at the 2-mg/kg level. As shown in figure 9, this trial includes provisions for escalations of the MTX dose.

SUMMARY OF DRUG VARIABLES IN CLINICAL PROTOCOLS

Table 2 is a summary table of the clinical protocols and shows the variables in the MTX-CF rescue regimens which were previously noted. The variability in the amount of MTX which is administered according to the different protocols and the lowest and highest dose of MTX employed in each of the protocols are shown in column 2 of this table. Overall, the amount of MTX administered ranges from 100 mg (study No. 1) to 600 mg/kg (study No. 9). Column 3 shows the time period over which a given amount of MTX is administered. Overall, this varies from 2 to 36 hours. In some studies, a lesser amount of MTX is given over a greater period of time. Also, in one study (No. 6, fig 5), 10% of the MTX dose is given as an intravenous (iv) push and the remaining 90% of the MTX is given as a 24-hour iv drip. Column 4 shows the interval between MTX injections when there is escalation from the initial amount to some higher amount. In those trials in which the MTX dose is escalated, there appears to be general agreement that a period of 2-3 weeks should be allowed to pass before escalating to the next highest dose level. The variability in how long to wait before initiating rescue with CF is shown in column 5. It is seen that this varies from no delay at all to a delay period of 6 hours. As indicated, there appears to be no correlation between the dose of MTX and the duration of time before initiating CF rescue. Column 6 shows the variability in duration of CF rescue and the dose of CF as compared to the amount of MTX given. In most of the protocols, the amount of CF to be given is dependent upon the body surface area or body weight of the patient. For severe toxicity, study No. 6 has a provision for administering CF as a continuous infusion at a dose of 50 mg every 8 hours. Finally, as shown in column 7 of table 2, the route of CF rescue is by oral administration, intramuscular (im) or iv injection, or a combination of oral and parenteral routes.

Still another variable with regard to CF rescue is provided by the *Physicians' Desk Reference* (1975). This source states that in general for the treatment of overdosage of MTX, CF (a) should be administered within the first hour, and is much less effective if

TABLE 2.—Summary of clinical protocols and variables in the MTX-CF rescue regimens

Study No.	Amount of high-dose MTX (lowest/highest)	Infusion time (hrs)	Interval between injections	Delay time (hrs) for CF rescue	Dose and schedule of CF rescue	Route of administration of CF rescue
1	100 mg/12 g	2-18	10 days-3 wks	1-6	6-25 mg q6h × 6-24.	Im
2	50/250 mg/kg	6	Every 2 wks up to 200 mg/kg; then every 3 wks	2	Schedule I: for MTX ≤100 mg/kg— a. <0.5 m^2: 6 mg q6h × 12. b. 0.5-1 m^2: 9 mg q6h × 12. c. >1 m^2: 12 mg q6h × 12.	Im
					Schedule II: for MTX ≥200 mg/kg— a. <0.5 m^2: 9 mg q6h × 12. b. 0.5-1 m^2: 12 mg q6h × 12. c. >1 m^2: 15 mg q6h × 12.	Im
3	240 mg/m^2/*	36	Not specified	Immediately	40 mg/m^2 over 6 hrs, then 25 mg q6h × 4.	Iv, oral
4	100/100 mg/kg	6	No escalation	3	5 mg/kg in 8 divided doses at 3-hr intervals for 24 hrs; wait 6 hrs, then 12 mg/m^2 q6h × 8.	Iv, im
5	100/300 mg/kg	6	2 wks	2	a. <0.5 m^2: 6 mg, then 3 mg q3h × 23. b. 0.5-1 m^2: 9 mg, then 4.5 mg q3h × 23. c. >1 m^2: 12 mg, then 6 mg q3h × 23.	Iv
6	75/250 mg/kg	24	Every 3 wks up to 150 mg/kg; then every 4 wks up to 250 mg/kg	6	a. <0.5 m^2: 6 mg q6h × 12. b. 0.5-1 m^2: 9 mg q6h × 12. c. >1 m^2: 12 mg q6h × 12. (duration of CF rescue will depend upon clinical developments)	Im
7	200/500 mg/kg	4	2-3 wks	4	9 mg q6h × 12.	Oral
8	75/150 mg/kg	24	48 and 28 days	6	a. <15 kg body wt: 6 mg q6h × 12. b. 5-30 kg body wt: 9 mg q6h × 12. c. >30 kg body wt: 12 mg q6h × 12.	Im
9	15/600 mg/kg	6	Every 2 wks up to 200 mg/kg; then every 3 wks up to 600 mg	2	Schedule I: for MTX ≤ 200 mg/kg— a. <0.5 m^2: 6 mg q6h × 12. b. 0.5-1 m^2: 9 mg q6h × 12. c. 1-2 m^2: 12 mg q6h × 12.	Im
					Schedule II: for MTX >200 mg/kg— a. <0.5 m^2: 9 mg q6h × 12. b. 0.5-1 m^2: 12 mg q6h × 12. c. 1-2 m^2: 15 mg q6h × 12.	Im
10	B† 50/50 mg/kg	4	No escalation	2	12 mg, wait 6 hrs, then 12 mg q6h × 11.	Iv, oral
	C† 200/200 mg/kg	4	No escalation	2	12 mg, wait 6 hrs, then 12 mg q6h × 11.	Iv, oral

*Not specified; increase dose by increments of 240 mg/m^2 until toxicity is reached.
†See fig 9.

administered after a delay of 1 hour, and (*b*) must be given in amounts equal to or higher than the offending dose of MTX.

AVAILABILITY OF MTX AND CF

MTX and CF, which are commercially marketed, are available from Lederle Laboratories, Pearl River, New York. In addition, three investigational dosage forms of MTX and one investigational dosage form of CF are available from the DCT, NCI. All investigational dosage forms of MTX from NCI are preservative-free. A description of the various dosage forms and their sources is shown in table 3.

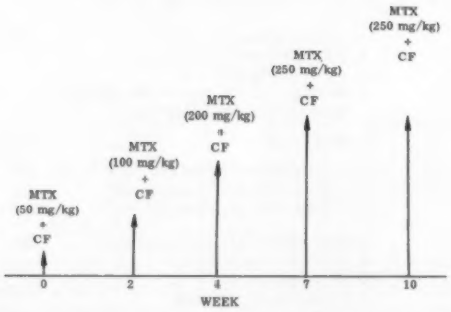
The clinical protocols which have been presented here, several of which represent the original work with high-dose MTX plus CF rescue, illustrate how these drugs are used with other chemotherapeutic agents, radiotherapy, and surgery. These protocols also serve to focus upon a number of drug variables to be considered in performing clinical trials with high-dose MTX plus CF rescue.

At this time there is no accepted agreement as to what dose of MTX is "high-dose MTX." The safety and efficacy of these protocols remains to be proved, and the drug regimens employed can be excessive and fatal.

TABLE 3.—Availability and description of MTX and CF dosage forms

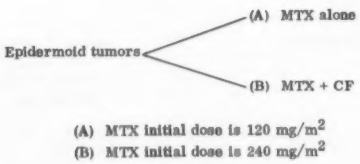
Drug and route of administration	Size/form	Concentration (mg/cc)	pH	Source
MTX, parenteral	5 mg/vial	2.5	8.5	Lederle Laboratories
	50 mg/vial	25	8.5	Lederle Laboratories
	50 mg/vial*	10	7.8-8.8	NCI
	500 mg/vial*	25	7.8-8.8	NCI
	1 g/vial*	50	7.8-8.8	NCI
CF, parenteral and oral	3 mg/ampule	3	7.6	Lederle Laboratories
	5 mg/tablet†	–	–	Lederle Laboratories
	50 mg/vial‡	10	6.3-7.3	NCI

*Freeze-dried investigational dosage forms which contain no preservatives.
†Investigational dosage form.
‡Freeze-dried investigational dosage form which contains preservatives.



1. Ablate tumor wherever possible and institute high-dose MTX shortly thereafter.
2. Vincristine (0.05 mg/kg iv, maximum 2 mg) is administered 1/2 hr before each MTX infusion.
3. MTX dose is escalated from 50 to 250 mg/kg; interval between injections is 2 wks for up to 200 mg/kg and then 3 wks before escalation to 250 mg/kg.
4. MTX is discontinued if excessive toxicity is encountered or if there has been failure of response after administration of 2 doses of 250 mg/kg.
5. For prophylaxis, a 2-yr period of treatment is projected.

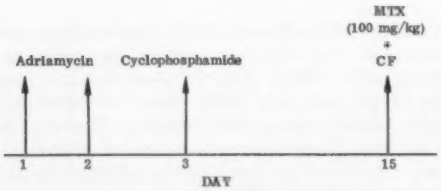
FIGURE 1.—Schema and synopsis of protocol used in study No. 2.



(A) MTX initial dose is 120 mg/m^2
(B) MTX initial dose is 240 mg/m^2

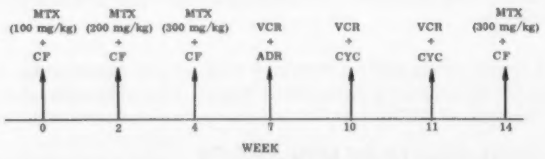
1. The object of the protocol is twofold:
   a. to determine if CF rescue increases the therapeutic index of high-dose MTX, and
   b. to correlate toxicity and tumor response with blood levels of drug.
2. The toxicity end-point is defined as 1 or more of the following:
   a. mucositis: grade 3+-4+;
   b. granulocyte count < 1500 cells/mm^3;
   c. platelet count < 100,000 platelets/mm^3;
   d. moderate to severe skin rash.
3. The dose escalation of MTX is not specified.
4. Patients reaching end-point toxicity without response are considered treatment failures. Patients are maintained at doses which are less than the dose which causes toxicity end-point to be reached.

FIGURE 2.—Schema and synopsis of protocol used in study No. 3.



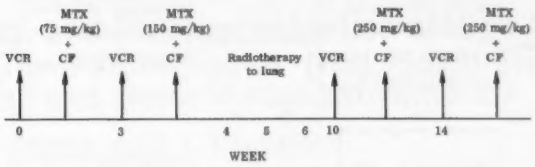
1. Chemotherapy starts within 1 mo after amputation.
2. Adriamycin = 25 mg/m^2/day × 2 iv and cyclophosphamide = 600 mg/m^2 iv.
3. No escalation in MTX dose.
4. Schedule of drugs is given monthly for 10 mos.

FIGURE 3.—Schema and synopsis of protocol used in study No. 4.



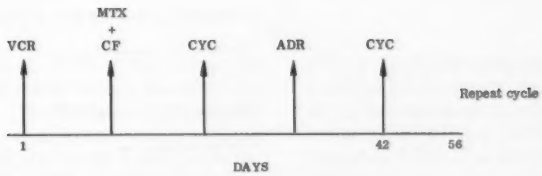
1. Surgery precedes chemotherapy.
2. (a) Vincristine (2 mg/m^2 iv, maximum 2 mg) and adriamycin (60 mg/m^2 iv) are administered 3 wks after 300-mg/kg MTX dose. (b) Vincristine (2 mg/m^2 iv, maximum 2 mg, on Days 1 and 7) and cyclophosphamide (15 mg/kg/day × 7 iv or orally) are started 3 wks after vincristine and adriamycin.
3. MTX dose is escalated from 100 to 300 mg/kg; interval between injections is 2 wks.
4. Rotation through series of all drugs is continued for 1 yr.

FIGURE 4.—Schema and synopsis of protocol used in study No. 5. VCR = vincristine; ADR = adriamycin; CYC = cyclophosphamide.



1. All patients who have metastatic (pulmonary) osteogenic sarcoma not amenable to surgical treatment will be eligible.
2. Vincristine (2 mg/m^2, maximum 2 mg) is given 24 hrs before each MTX infusion.
3. Ten percent of MTX dose is given as iv push; 90% of MTX dose is given as 24-hr iv drip.
4. MTX doses will be reduced in 50-mg/kg decrements if severe toxicity recurs despite increased CF doses.
5. For severe toxicity, iv CF may be given q3h x 24 but using 1/2 of the calculated im dose. Also, CF may be given as a continuous infusion--50 mg q8h.
6. Maintenance therapy with vincristine-MTX (250 mg/kg) is continued q4wks until relapse or for 2 yrs from completion of radiotherapy.

FIGURE 5.—Schema and synopsis of protocol used in study No. 6. VCR = vincristine.



Repeat cycle 7 times within 1 yr.

FIGURE 6.—Protocol schema of study No. 7. VCR = vincristine; ADR = adriamycin; CYC = cyclophosphamide.

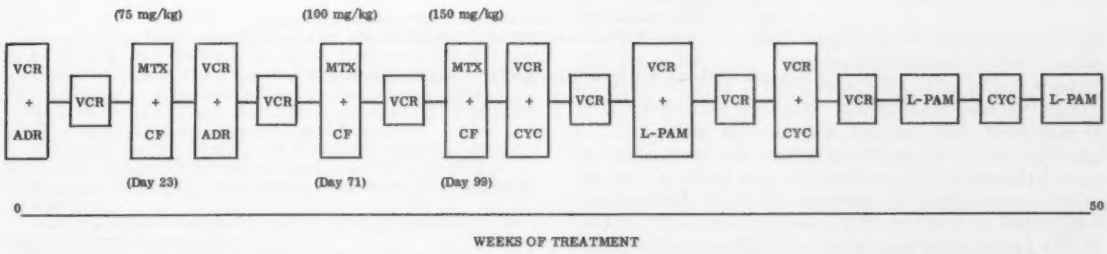
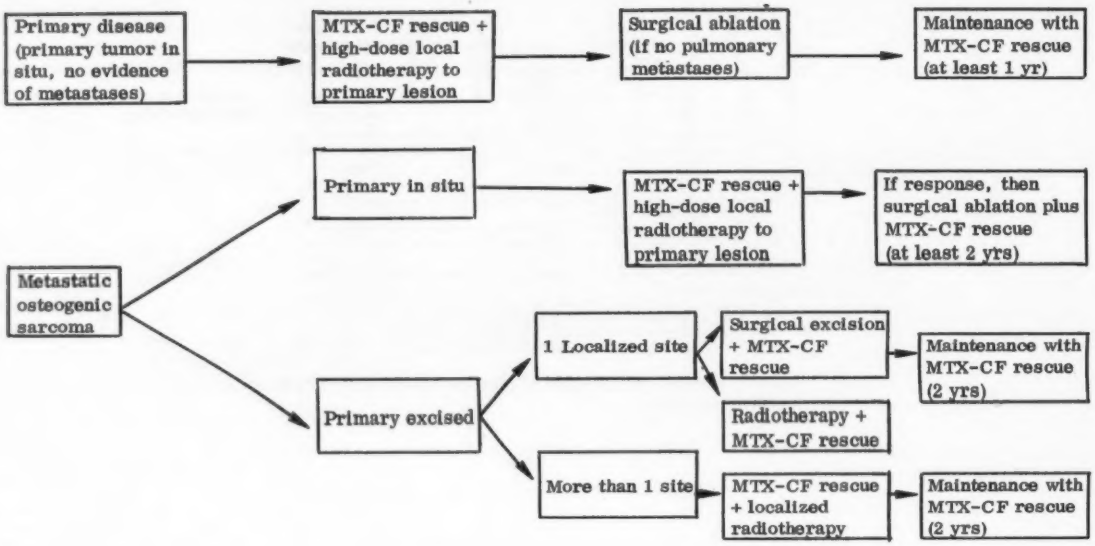
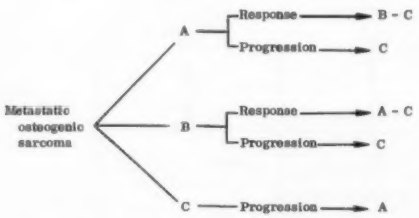


FIGURE 7.—Outline of Compadri-II study (No. 8). VCR = vincristine; ADR = adriamycin; CYC = cyclophosphamide; L-PAM = melphalan.


Primary disease (primary tumor in situ, no evidence of metastases)
MTX-CF rescue + high-dose local radiotherapy to primary lesion
Surgical ablation (if no pulmonary metastases)
Maintenance with MTX-CF rescue (at least 1 yr)
Metastatic osteogenic sarcoma
Primary in situ
MTX-CF rescue + high-dose local radiotherapy to primary lesion
If response, then surgical ablation plus MTX-CF rescue (at least 2 yrs)
Primary excised
1 Localized site
Surgical excision + MTX-CF rescue
Maintenance with MTX-CF rescue (2 yrs)
Radiotherapy + MTX-CF rescue
More than 1 site
MTX-CF rescue + localized radiotherapy
Maintenance with MTX-CF rescue (2 yrs)

Each time MTX is given, patients are escalated from 15 to 600 mg/kg or less, depending upon toxicity.

FIGURE 8.—Schema and synopsis of protocol used in study No. 9.



A = Small-dose MTX without CF rescue; initial MTX, 2 mg/kg
B = Low-dose MTX + CF rescue; initial MTX, 50 mg/kg
C = High-dose MTX + CF rescue; initial MTX, 200 mg/kg

1. Schedules A, B, and C will be given every 2 or 3 wks x 4 unless obvious clinical deterioration occurs.
2. After completion of 4 treatment cycles, patients are classified into 4 categories:
   A. Stable, no toxicity; increase dose by 20% increments until tolerable toxicity is achieved. Therapy is continued every 2 or 3 wks x 8, then progresses to an every 3- or 4-wk schedule.
   B. Stable, tolerable toxicity; maintain constant dose; therapy is continued every 2 or 3 wks x 8, then progresses to an every 3- or 4-wk schedule.
   C. Responders: same as B.
   D. Disease progression: *see* schema above.
3. On all 3 schedules, dosages must be reduced by 33% if more than tolerable toxicity is seen at any time. This includes anything more than mild diarrhea.

FIGURE 9.—Schema and synopsis of protocol used in study No. 10.

Response, Toxicity, and Pharmacokinetics of High-Dose Methotrexate (NSC-740) With Citrovorum Factor (NSC-3590) Rescue for Children With Osteosarcoma and Other Malignant Tumors[1,2]

C. B. Pratt, D. Roberts, E. Shanks, and E. L. Warmath[3,4]

SUMMARY

Methotrexate (50-500 mg/kg) was administered by a 6-hour infusion to 46 children with malignant tumors. Citrovorum factor rescue was started 3 hours after the methotrexate infusion and continued at 3-6-hour intervals for 24-72 hours. Sixteen patients received methotrexate infusions with citrovorum factor rescue and adriamycin plus cyclophosphamide as adjuvant therapy after an amputation for osteosarcoma. At the termination of drug infusion, serum levels ranged between 71 and 727 μg/ml of methotrexate with drug dosages of 100 and 500 mg/kg, respectively. The mean half-life $\pm$standard deviation of the serum methotrexate was 157 $\pm$31 minutes. Urinary excretion during the 6 hours of drug infusion ranged between 35% and 53% of the administered dose. Ninety-five percent of the drug was excreted in the first 30 hours after the initiation of the drug infusion. From the present experience with three different treatment protocols we can recommend high-dose methotrexate with citrovorum factor rescue only for the treatment of osteosarcoma. Currently, we favor the administration of methotrexate (100 mg/kg) by a 6-hour infusion followed 3 hours later by intravenous citrovorum factor in a total dose of 5 mg/kg divided into eight equal doses and thereafter the administration of eight intramuscular doses of 12 mg/m^2 of citrovorum factor at 6-hour intervals.

[Cancer Chemother Rep Part 3, vol 6:13-18, 1975]

Since their introduction in 1948, many changes have been made in the treatment schedules used for the folic acid antagonists (1). Methotrexate (MTX) was initially used on a daily oral treatment schedule. Improved response followed the twice weekly administration of larger oral, intramuscular (im), or intravenous (iv) doses (2-4). Once weekly treatment schedules were also effective, and intermittent treatment with large doses of MTX induced remission of acute lymphocytic leukemia that was resistant or insensitive to conventional dosages (5,6). Elevation of MTX levels in the serum and consequently in the tumor cell was the aim of these various treatment protocols (7-9). Thus, high-dose MTX treatment with citrovorum factor (CF) rescue was a logical extension of earlier preclinical and clinical studies (10-14).

Our experience with the administration of 177 high-dose MTX infusions with CF rescue to 46 children with malignant tumors including osteosarcoma and the preliminary results of a comparative study of high versus conventional dosages of MTX given to children with a variety of malignant tumors are reported here. The serum and urine MTX levels after high-dose infusions are also reported.

MATERIALS AND METHODS

Patients and Treatment

Nine children, 5-17 years of age with drug-resistant malignant neoplasms (four osteosarcoma, two rhabdomyosarcoma, one liposarcoma, one Ewing's sarcoma, and one mixed malignant germ cell tumor of the ovary), were treated with 31 courses of MTX in dosages of 50-500 mg/kg, followed by 2-40 mg/kg of CF rescue given over the 30-hour period after the infusion of MTX (11).

MTX was supplied in sterile ampules containing 500 mg. The agent was administered in increasing dosages at 2- or 3-week intervals. The initial dosage of MTX was 50 mg/kg for one patient and 100 mg/kg for the other 45 patients. Dosage was increased in increments of 100 mg/kg to a final dosage of 500 mg/kg and the drug was administered by 6-hour infusion in 500-1000 ml of 5% dextrose. Iv fluids were continued for the next 24 hours. CF rescue was initiated 3 hours after the MTX infusion was terminated. CF (2-40 mg/kg) divided into eight doses was administered iv at 3-hour intervals.

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[1]Supported by Public Health Service grants CA-08480, CA-11148, and CA-12732 from the National Cancer Institute (NCI), National Institutes of Health, Department of Health, Education, and Welfare; and the American Lebanese Syrian Associated Charities.

[2]Methotrexate: CAS reg. No. 59-05-2; glutamic acid, *N*-[*p*-[[(2,4-diamino-6-pteridinyl)methyl]methylamino]benzoyl]-.

Citrovorum factor: CAS reg. No. 6035-86-5; glutamic acid, *N*-[*p*-[[(2-amino-5-formyl-5,6,7,8-tetrahydro-4-hydroxy-6-pteridinyl)-methyl]amino]benzoyl]-, calcium salt; leucovorin; N^5-formyltetrahydrofolate.

Both drugs were supplied by Cancer Therapy Evaluation, Division of Cancer Treatment, NCI.

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In a comparative study of the efficacy of each treatment schedule, 30 children and young adults ranging in age from 1 to 22 years, whose tumors were refractory to conventional chemotherapeutic agents, received either 25 mg/m^2 of MTX twice weekly or 50-200 mg/kg of MTX by 6-hour infusion followed by 2.5-10 mg/kg of CF. Thirteen of these 30 patients received intermittent treatment with high-dose infusions at 2-week intervals.

Twenty-four children with osteosarcoma (16 clinically localized, two regional, and six metastatic) received two to 11 high-dose MTX infusions followed by CF rescue alternating with adriamycin and cyclophosphamide[5] as adjuvant chemotherapy or for the treatment of regional or metastatic osteosarcoma. For patients with clinically localized tumor, doses of MTX (100 mg/kg) were given followed by iv CF rescue (5 mg/kg) given in eight divided doses at 3-hour intervals over a 24-hour period. Im CF (12 mg/m^2) was started 6 hours later and continued at 6-hour intervals for eight doses. For patients with regional and metastatic osteosarcoma, dosages of MTX were increased in 100-mg/kg increments to 300 mg/kg. Iv CF in dosages equivalent to 5% of the MTX dose was administered in eight divided doses over a 24-hour period followed by im CF rescue in a dosage of 12 mg/m^2 at 6-hour intervals for 48 hours.

Serum and Urine MTX Levels

Serum and urine levels of MTX were assayed by inhibition of dihydrofolate reductase (15). Rat liver was the source of dihydrofolate reductase and a nicotinamide adenine dinucleotide phosphate reduced (NADPH)-regenerating system for the assay. A 25% rat liver homogenate in 0.25 M sucrose/2 mM tris chloride, pH 7.0, was prepared by centrifugation at 25,000 × *g* for 30 minutes. Reagent for the assay contained 0.46 M dimethylglutarate, sodium (pH 6.1); 46 μM $MgCl_2$; 61 μM citrate; 1mM NADPH; 1.1 μM folate; and 0.15 microcurie/ml of generally labeled H^3-folate (16). Serum was serially diluted with 0.07 M dimethylglutarate, pH 6.1, to a drug concentration that resulted in approximately 50% inhibition of the enzyme. A standard titration curve of MTX inhibition was prepared simultaneously with the assay of serum or urine specimens. The serum or urine content of MTX was determined from the standard titration curve. MTX standards were prepared from the same lot of drug that was administered. The incubation system contained 23.1 μl of diluted serum, urine, or MTX standard, 7.9 μl of reagent, and 16.3 μl of rat liver homogenate. After 1 hour of incubation at 37°C, 47 μl of 15% trichloroacetic acid containing 0.05% $NaNO_2$ was added. After 10 minutes at room temperature, 77.6 μl of charcoal suspension (100 mg/ml) was added, and the mixture was centrifuged for 10 minutes at 500 × *g*. A 79.3-μl aliquot of the supernatant fluid was diluted in 10 ml of liquid scintillation cocktail for counting (16). Serum levels were determined for seven patients, and three patients had these determinations done twice. Blood was collected immediately before the 6-hour MTX infusion was initiated to test for nonspecific inhibition (which was not observed) and at 3-hour intervals for 24 hours after completion of the infusion. In an attempt to avoid nonspecific inhibition by supportive therapy, all serum specimens were diluted at least 100-fold before the assay. Urinary excretion was assayed after six MTX infusions. For 30 hours after initiation of the MTX infusion, urine was collected and pooled for 3-hour intervals.

RESULTS

Therapeutic Response

Objective responses to 31 high-dose MTX infusions with CF rescue for nine children with various tumors were observed after treatment with 100 mg/kg of MTX (14). Transient disappearance of pulmonary metastases was observed in one of four patients with osteosarcoma. A partial response of pulmonary metastases of rhabdomyosarcoma was observed in one patient, while the size of the primary tumor decreased without change in the pulmonary metastases for a patient with perineal rhabdomyosarcoma.

The tumor responses after twice weekly iv MTX or biweekly infusions of 100-200 mg/kg of MTX with CF rescue are outlined in table 1. A partial response was observed in one of two patients with histiocytic lymphoma and in one of two patients with embryonal carcinoma after twice weekly injections of 25 mg/m^2 of MTX. After high-dose infusion of MTX, a mixed response occurred in one child with generalized neuroblastoma and in one child with nodal metastases of Wilms' tumor.

Of 16 patients with clinically localized osteosarcoma who received monthly adjuvant chemotherapy with adriamycin and cyclophosphamide plus high-dose MTX and CF rescue 2 weeks later, 14 have remained clinically well and free of metastatic tumor for 2-24 months after surgery. Two patients with primary regional osteosarcoma of the scapula and of the zygoma had no progression of tumor for 2 and 3 months respectively with this multiple combination chemotherapy. Of the six patients with metastatic osteosarcoma, one had a complete response of 5 months' duration, one had no evidence of pulmonary metastases for 6 months after amputation and wedge resection of a single pulmonary metastasis, one had a partial response of pulmonary and nodal metastases, one has stable pulmonary and bony metastases for more than 4 months, and two patients had progressive disease.

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[5]Adriamycin: NSC-123127; CAS reg. No. 23214-92-8.
Cyclophosphamide: NSC-26271; CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)amino]tetrahydro-, 2-oxide, monohydrate.

TABLE 1.—Objective responses after low- and high-dose MTX

Tumor	No. of patients	Responses with MTX treatment schedule of—* 25 mg/m² twice weekly	100-200 mg/kg iv (6-hr infusion)†
Rhabdomyosarcoma	9	5 NR, 1NE	3NR
Neuroblastoma	8	3 NR	1 MR, 4 NR
Histiocytic lymphoma	2	1 PR, 1 NR	
Embryonal carcinoma	2	1 MR, 1 NR	
Wilms'	2	1 MR	1 MR
Ewing's sarcoma	2	1 NR	1 NR
Osteosarcoma	1	1 NR	
Carcinoma of nasopharynx	1	1 NR	
Hemangiopericytoma	1		1NR
Carcinoma of adrenal gland	1		1 NR
Neurofibrosarcoma	1		1 NR

*PR = partial response; MR = mixed response; NR = no response; NE = not evaluable.
†Plus 5 mg/kg of CF in 8 divided doses.

TABLE 2.—Incidence of hematologic, hepatic, and renal function abnormalities after high-dose MTX infusions

Iv dose of—			No. of infusions resulting in abnormalities of—		
MTX (mg/kg)	CF* (mg/kg)	No. of infusions	Hematologic function	Hepatic function	Renal function
50	2-2.5	3	0	0	1
100	10	11	2	3	1
100	5†	126	12	3	5
200	10-20	26	2	12	3
300	15-30	5	2	2	0
400	20-40	4	2	4	2
500	25-30	2	0	2	0

*Administered in 8 divided doses at 3-hr intervals.
†Followed by 12 mg/m² im every 6 hrs for 48 hrs.

The observed incidence of hematologic, hepatic, and kidney toxicity after treatment with various dosages of MTX-CF rescue is listed in table 2. Life-threatening hematologic abnormalities were seen in three patients who received 100 mg/kg of MTX followed by 5 mg/kg of CF in eight divided iv doses. Other patients had minor depression of hemoglobin levels and white blood cell and platelet counts. Transient elevation of serum glutamic oxaloacetic transaminase (SGOT) levels occurred after infusions of MTX; these elevated values were usually associated with the administration of 200 mg/kg or more of MTX and did not persist for more than 7 days. Renal function abnormalities consisted of minor increases of serum uric acid levels and blood urea nitrogen values; creatinine clearance values were not abnormal except in one patient with pretreatment evidence of hydronephrosis. One patient died 17 days after her third infusion of 100 mg/kg of MTX; toxicity included exfoliative dermatitis, severe myelosuppression with megaloblastic features, hepatic and renal failure, and *Escherichia coli* and *Candida tropicalis* septicemia. Two patients who were treated more than 6 months after having a nephrectomy for Wilms' tumor and adrenocortical carcinoma tolerated treatment with 100 mg/kg of MTX plus CF rescue without evidence of MTX toxicity.

Serum and Urine MTX Levels

Higher serum levels were found to be associated with higher dosages of MTX (table 3). Serum levels varied between 71 and 727 μg/ml at termination of MTX infusion. Forty-one percent of the MTX dose (range 35%-53% for four infusions) was excreted by

the time that the infusion was completed at 6 hours (table 4).

After completion of the infusion, MTX disappeared from the serum with a mean half-life ± standard deviation of 157 ± 31 minutes (table 3). With the serum concentrations found for these patients, the disappearance of MTX was monophasic and the initiation of CF rescue after collection of the 9-hour specimen did not change the rate of the disappearance of MTX from the serum (fig 1).

MTX is excreted primarily via the kidneys (17). Drug recovery from urine in the first 30 hours after the completion of the MTX infusion averaged 95% for six patients and ranged from 77% to 110% of the administered drug.

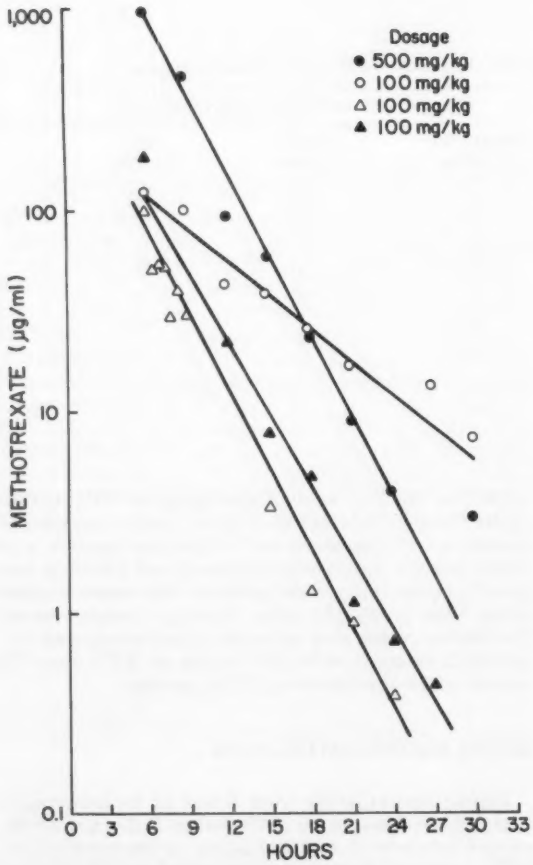

FIGURE 1.—Serum levels of MTX after a 6-hr infusion.

DISCUSSION

When nausea and vomiting were observed after large injections of CF, the dosage of CF was reduced from 10% to 5% by weight of the MTX dose. After the death of one patient with classic signs of MTX toxicity, CF rescue was extended to 72 hours by the addition of eight im injections. Prolongation of the CF rescue also has reduced the incidence of mucosal ulcerations and hematopoietic suppression.[6]

MTX, a folic acid antagonist, is a potent inhibitor of dihydrofolate reductase, an enzyme which catalyzes the reduction of folate or dihydrofolate to form tetrahydrofolate (18,19). By inhibiting tetrahydrofolate synthesis, MTX decreases formation of 1-carbon units for synthesis of purine nucleotides and thymidylic acid, thus inhibiting DNA synthesis and cell replication.

CF, when administered after MTX, has prevented the toxicity of MTX without reversing the oncolytic effect of MTX (10-12,20,21). This protection from potentially lethal doses of MTX has been called "CF rescue" (18). CF administration provides tetrahydrofolate as well as a 1-carbon unit for MTX-inhibited cells, thus protecting normal cells until MTX is excreted and adequate metabolism is recovered.

Our studies showing a serum half-life of 2-3.5 hours for the disappearance of MTX are in agreement with the reported values of 134 ± 21 minutes for the initial rapid phase of MTX disappearance after more conventional dosages (22). MTX excretion did not appear to be altered by administration of CF over a period of 30 hours after the infusion of MTX.

The fall in serum levels of drug obeyed first-order kinetics. The half-life value of MTX correlates with the "initial" phase observed by Johns et al (23). Any slow displacement of drug from tissues presumably developed after 30 hours. Our studies failed to indicate the third phase (half-life = 45 minutes) observed by Huffman et al (24).

With the exception of osteosarcoma, the response of childhood malignant tumors has not justified continuation of the studies of treatment with MTX. The moderate toxicity and therapeutic success encountered with the present treatment schedules do warrant the continuation of treatment with MTX for osteosarcoma, particularly as part of adjuvant chemotherapy for this tumor (25,26). We currently favor treatment with 100 mg/kg of MTX by a 6-hour infusion followed 3 hours later by iv CF rescue in a total dose of 5 mg/kg divided into eight equal doses and thereafter into eight im doses of 12 mg/m² of CF at 6-hour intervals.

[6]Pratt CB, and Shanks EC. Unpublished observations.

TABLE 3.—Levels and rate of disappearance of MTX from serum*

Patient No.	Dosage (mg/kg)	Serum level (μg/ml) at—		Half-life† (min)
		6 hrs	30 hrs	
1	100	130	7.3	195
2	100	71	‡	125
3	100	108	‡	125
4	100	132	0.04	165
5	100	132	0.1	150
6	200	257	0.03	210
7	300	339	‡	160
8	400	404	3.0	165
9	500	727	2.5	120

*CF rescue was initiated 3 hrs after termination of MTX infusion.
†Mean half-life for disappearance was 157 ± 31 min, mean ± SD.
‡MTX content of a 1:100 serum dilution was below the sensitivity of the assay.

TABLE 4.—Urinary excretion of MTX during 6-hr drug infusion

Patient No.	Amount of MTX (g)—		Recovered (%)
	Infused	Excreted	
6	9.78	4.34	44
7	14.16	5.10	35
9	14.00	7.40	53
5	5.07	2.27	45

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Pharmacokinetics of High-Dose Methotrexate (NSC-740)[1]

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SUMMARY

High-dose methotrexate (MTX) therapy with citrovorum factor (CF) rescue, as described by Jaffe et al, is occasionally associated with renal failure, myelosuppression, and gastrointestinal toxicity. In an effort to understand the reasons for this toxicity, the pharmacokinetics of the Jaffe regimen were studied in six patients during seven infusions. Peak serum MTX during infusion was proportional to dose: 0.1-0.3 mM for 50 mg/kg, 0.48-0.63 mM for 100 mg/kg, and 1.0 mM for 200 mg/kg. Two phases of plasma disappearance were seen after infusion in nontoxic patients; the first phase had a half-life of 2.06 ± 0.16 hours and the second slower phase had a half-life of 10.4 ± 1.8 hours. In these nontoxic infusions, cumulative urinary excretion in the first 12 hours was greater than 50% of the administered dose. Peak urine levels were tenfold greater than peak plasma MTX and routinely exceeded the 2 mM maximum solubility of MTX in urine at pH 5.5 or below. In one patient with urine pH 5.0-5.5 during the infusion, azotemia resulted within 24 hours of starting MTX; plasma MTX remained above 10^{-7} M for 16 days and severe myelosuppression and stomatitis occurred. The high urinary MTX levels resulting from high-dose MTX therapy and the limited solubility of MTX in acid urine indicate the need for urinary alkalinization and high urine volume to avert MTX precipitation in the renal tubules, azotemia, and delayed drug excretion.

[Cancer Chemother Rep Part 3, vol 6:19-24, 1975]

Recent reports of success in the treatment of osteogenic sarcoma using high-dose methotrexate (MTX) with citrovorum factor (CF)[5] rescue (1,2), have stimulated new interest in the pharmacology and biochemistry of this agent. The rationale for such therapy has been to enhance the intracellular concentration of the drug while preventing excessive toxicity to host tissues by the use of the antidote, CF. Previous studies have shown that MTX infusions continued for more than 30 hours were usually associated with significant toxicity to the bone marrow and gastrointestinal tract (3,4), while shorter durations of infusion were well tolerated. However, Frei et al (5), Djerassi et al (6), and others (7) have reported that infusions of less than 30 hours may be accompanied by seemingly unpredictable myelosuppression and renal failure, particularly in adults.

The present investigation of MTX pharmacokinetics was undertaken to determine the characteristics of plasma disappearance and urinary excretion in a group of patients receiving 50-200-mg/kg infusions and to examine the deviations in these kinetic parameters associated with clinical toxicity.

MATERIALS AND METHODS

Patients

Six patients, ages 11-48 years, were selected for high-dose MTX therapy (table 1). Routine hematologic and chemical studies including complete blood count (cbc), urinalysis, blood urea nitrogen (BUN), creatinine, serum glutamic oxaloacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT), and bilirubin were normal in all patients prior to the study period and were monitored for at least 3 days following the infusion.

Treatment Regimen

MTX was given according to the Jaffe regimen (2). Doses of 50-200 mg/kg were infused intravenously (iv) over 6 hours followed by CF rescue. CF (15 mg/m^2) was given iv every 6 hours for eight doses, beginning 2 hours after completion of the MTX infusion. In order to ensure a high urine volume and an alkaline urine, iv fluids were administered at a rate of 3 liters/m^2/24 hours with 25 mEq of $NaHCO_3$/liter, beginning 12 hours prior to the MTX infusion and continuing for 24 hours thereafter.

MTX Levels

Blood samples were collected in heparin and the plasma was immediately separated and stored at 4°C. Urine was collected in 6-hour fractions for the first 24 hours, and then in 24-hour fractions for 2 or

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[1] Methotrexate: CAS reg. No. 59-05-2; glutamic acid, *N*-[*p*-[[(2,4-diamino-6-pteridinyl)methyl]methylamino]benzoyl]-; MTX.

[2] Laboratory of Chemical Pharmacology (LCP), Experimental Therapeutics (EX), Division of Cancer Treatment (DCT), National Cancer Institute (NCI), Bethesda, Md.

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[5] Citrovorum factor: NSC-3590; CAS reg. No. 6035-86-5; glutamic acid, *N*-[*p*-[[(2-amino-5-formyl-5,6,7,8-tetrahydro-4-hydroxy-6-pteridinyl)methyl]amino]benzoyl]-, calcium salt; leucovorin.

TABLE 1.—Pharmacokinetics of high-dose MTX: plasma clearance

Patient No.	Age (yrs)	Diagnosis	Dose (mg/kg)	Peak plasma MTX (mM)	First half-life* (hrs)	Second half-life† (hrs)
1	32	Osteogenic sarcoma	50	0.30	2.16	9.75
2	35	Osteogenic sarcoma	50	0.10	1.75	12.69
3	48	Epidermoid carcinoma, lung	50	0.19	360‡	64‡
4	29	Osteogenic sarcoma	50	0.09	2.10	8.14
5	31	Osteogenic sarcoma	100	0.63	2.15	9.52
6	32	Osteogenic sarcoma	100	0.48	2.17	9.57
7	11	Osteogenic sarcoma	200	1.00	2.03	12.49

*Mean half-life = 2.06 ± 0.16 min for exponencial plasma decline following initial cellular distribution phase.

†Mean half-life = 10.4 ± 1.8 hrs for second-phase half-life.

‡Half-life determinations for patient No. 3 are excluded because of renal failure.

more additional days. Serum and urine MTX levels were determined by the dihydrofolate reductase method of Bertino and Fischer (8), using dihydrofolate reductase from MTX-resistant murine L1210 leukemia cells. Absorption spectra on urine samples collected during the initial 24 hours, diluted at least 1:50 in 1% $NaHCO_3$, were determined using the Cary 15 recording spectrophotometer.

Calculation of Plasma Half-Life

A nonlinear least squares regression program was used to fit a biexponential curve to the plasma decay data, and was based on the formula

$$Cp - a_1 e^{-b_1 t} + a_2 e^{-b_2 t}$$

where *Cp* is the MTX plasma concentration as a function of time and a_1, a_2, b_1, and b_2 are constants determined by the regression analysis. Half-lives for the biphasic plasma decay curve were calculated from each constant *b* according to the following equation:

$$t\frac{1}{2} = \frac{0.693}{b}$$

Results

The data obtained during the six nontoxic infusions are presented in the following sections. The single infusion (patient No. 3, 50 mg/kg) associated with severe renal, bone marrow, and gastrointestinal toxicity is discussed separately (*see* Toxicity).

Plasma MTX

Peak plasma levels were attained during the 6-hour period of infusion and were proportional to the dose administered (table 1). Mean peak levels were 0.168 mM, 0.553 mM, and 1.0 mM for doses of 50, 100, and 200 mg/kg respectively. Plasma levels fell rapidly following the infusion, with an initial mean plasma half-life of 2.06 ± 0.16 hours. A slower final phase of plasma clearance (half-life of 10.4 ± 1.8 hours), as previously described at conventional doses (9), was detected in each of six infusions, and began at least 30 hours after termination of the infusion and at plasma levels below 1×10^{-7} M (fig 1).

Urinary MTX

Peak urinary levels were approximately tenfold greater than peak plasma levels, ranging from 1.7 to 11 mM, and were attained in all patients during the first 12 hours of urine collection. Cumulative urinary MTX excretion during the initial 12 hours exceeded 50% of the administered dose in five of the six infusions (table 2). In the single patient (No. 6) with no toxicity where urine excretion fell below 50% of the administered dose, urine collection was incomplete for the 6-12-hour period, and 33% of the administered dose was excreted in the initial 6 hours. Urinary pH was kept at or above 7.0 in all cases unassociated with clinical toxicity, and the mean urinary volume for the first 12 hours was 3376 ml.

TABLE 2.—Pharmacokinetics of high-dose MTX: urinary excretion

Patient No.	Dose (mg/kg)	MTX excreted in initial 12 hrs		Peak urine MTX (mM)	Lowest urinary pH in initial 12 hrs	Urine volume in initial 12 hrs (ml)
		g	% of total dose			
1	50	2.65	75	2.3	7.0	2990
2	50	1.31	52	2.1	7.0	2460
3	50	0.66	21	3.7	5.0	450
4	50	3.90	82	2.3	7.0	5920
5	100	8.49	106	5.1	Not done	4820
6	100	2.69	38	3.0	7.0	2540
7	200	6.46	80	11.0	7.0	1415

*6-12-Hr urine collection incomplete.

Urine collected during the initial 12 hours of the treatment course had an ultraviolet spectrum closely approximating that of MTX, with peaks at 370, 302, and 257 nm at pH 7. However, in the 12-18-hour urine collections, a spectral change was noted in all patients, with plateauing of the 370-nm peak and a leftward shift of the 370- and 302-nm peaks (fig 2). While this new spectrum is compatible with the absorption characteristics of CF, preliminary studies suggest that this compound has dihydrofolate reductase-inhibiting properties and is therefore unlikely to be a naturally occurring reduced folate.

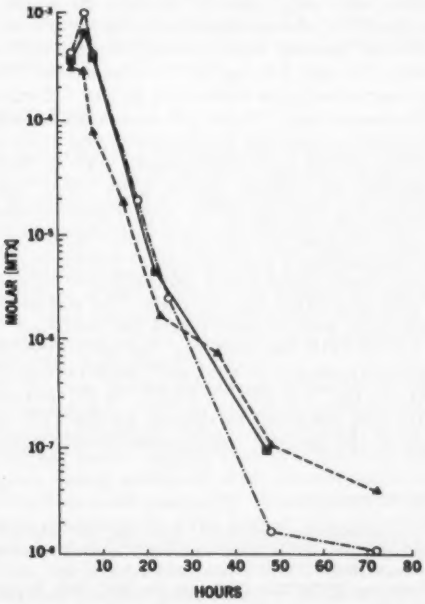

FIGURE 1.—Plasma MTX clearance curves for patient No. 7 (200 mg/kg, ○--○), No. 5 (100 mg/kg, ■——■), and No. 1 (50 mg/kg, ▲– –▲).

MTX Solubility in Urine: Relationship to pH

A heavy yellow precipitate was noted in urine in patients receiving high-dose MTX without urinary alkalinization, and this observation prompted further investigation of the relationship of MTX solubility to urinary pH. In urine specimens collected during MTX infusion, precipitation was readily produced in vitro by lowering the pH below 7.0 (table 3). Above pH 7.0, MTX remained in solution at concentrations greater than 20 mM, while the maximum solubility below neutrality decreased sharply, approaching a low value of 2 mM at pH 5.5. The precipitate formed by lowering the pH of 0- to 12-hour specimens was separated from the supernatant by centrifugation and was resuspended in 0.1 M tris-Cl (pH 7.5), and it proved to be MTX by spectrophotometric analysis and by dihydrofolate reductase inhibition assay. The primary clinical implication of these findings is that alkalinization of urine would appear to be mandatory to prevent precipitation of MTX in the urine during the high-dose infusion period, when the drug concentration in urine routinely exceeds 2 mM. Precipitation of MTX in the renal tubule is likely to occur at acid pH, and may be the mechanism of MTX-induced renal insufficiency.

TABLE 3.—Solubility of MTX in urine*

Urine pH	MTX remaining in solution (mM)
6.9	22
6.7	11
6.4	10
6.3	9.0
5.7	2.2

*Urine specimens were adjusted to indicated pH with 1 N HCl. An MTX precipitate was present in all samples at the indicated pH. The samples were equilibrated for 2 hrs at 37°C, the pH was re-measured, and the concentration of MTX remaining in solution was determined by ultraviolet absorption at 370 nm.

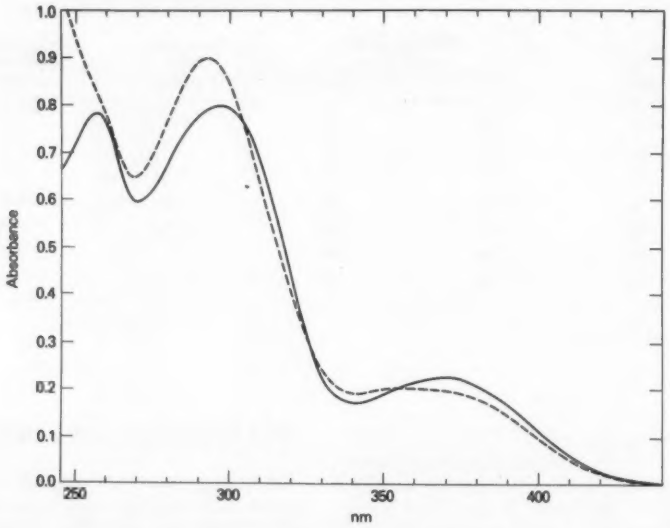

FIGURE 2.—Ultraviolet spectra of urine collected during the 0-6 hr (——) and 12-18-hr (——) collection.

Toxicity

In general, high-dose MTX with CF rescue was well tolerated, causing only mild nausea and vomiting easily controlled by antiemetics. Transient elevations of serum transaminases (one to four times the normal values) were seen after four of seven infusions, and in all cases these values returned to normal within 2 weeks. In six of seven infusions monitored in this study, there was no major bone marrow, mucous membrane, or renal toxicity. However, one patient did experience severe renal impairment associated with subsequent gastrointestinal and bone marrow toxicity, and his case will be described in detail.

Case Report

Patient No. 3, a 48-year-old white man with epidermoid carcinoma of the lung, was admitted to the National Cancer Institute on December 20, 1974, for high-dose MTX therapy after having failed to respond to therapy with cyclophosphamide, vincristine, adriamycin, bleomycin, and phase I gallium nitrate.[6] He had tolerated previous 6-hour infusions of MTX (250 and 600 mg/m^2) with CF rescue with no toxicity but with only subjective clinical improvement. Prior to this course of therapy his BUN, creatinine, SGOT, SGPT, bilirubin, alkaline phosphatase, and cbc were all within normal limits. Twenty-four-hour creatinine clearance was 62 ml/minute. MTX (50 mg/kg) was infused over 6 hours with urinary pH measurements of 5.0 and 5.5 during the infusion, although 25 mEq of $NaHCO_3$ was given prior to and 100 mEq was given during the infusion. Urine output during the first 12 hours after beginning the infusion was only 450 ml. Within 24 hours after starting MTX, the serum creatinine level rose to 2.6 mg/100 ml (normal = 0.7-1.4 mg/100 ml) and ranged between 2.3 and 3.4 mg/100 ml thereafter. Twenty-four-hour creatinine clearance fell to 7.2 ml/minute on the second day. Urine pH was kept between 6.3 and 7.5 with iv sodium bicarbonate, and urine output was kept above 1100 ml/24 hours with iv fluids and furosemide as needed. Seven days after the MTX infusion, the plasma MTX concentration was still 6.7×10^{-7} M, and the patient's white blood cell count (wbc) had fallen to 1700 cells/mm^3 (hemoglobin, 8.1 g/100 ml). The platelet count fell to 23,000 cells/mm^3 on the 13th day after the infusion. The patient's wbc, hemoglobin, and platelet count had returned to normal by the 19th hospital day. Serum transaminases peaked transiently on the tenth hospital day (SGOT, 84 IU [normal = 6-52 IU]; SGPT, 70 IU [normal = 2-45 IU]) and returned to normal by the 13th day. In addition, the patient experienced severe stomatitis

[6] Cyclophosphamide: NSC-26271; CAS reg. No. 6055-19-2; 2*H*-1,3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)amino]tetrahydro-, 2-oxide, monohydrate.
Vincristine: NSC-67574; CAS reg. No. 2068-78-2; leurocristine, sulfate (1:1).
Adriamycin: NSC-123127; CAS reg. No. 23214-92-8.
Bleomycin: NSC-125066.
Gallium nitrate: NSC-15200; CAS reg. No. 13494-90-1.

beginning on the 13th hospital day. In view of the significant toxicity and the patient's persistently elevated plasma MTX levels, CF was continued until the plasma MTX level fell below 10^{-8} M on the 22nd day after the MTX infusion. The patient received a total of 1342 mg of CF (632 mg iv; 360 orally; 350 intramuscularly). On the 28th hospital day, the patient died with progressive epidermoid carcinoma. Permission to perform an autopsy was not given.

The urinary and plasma pharmacokinetics of MTX in this patient (No. 3) differed markedly from the pattern seen in the six nontoxic MTX infusions. The plasma disappearance levels of MTX in this patient and in three nontoxic infusions at the same dose level (50 mg/kg) are shown in figure 3. These curves demonstrate a half-life of 360 minutes for the initial phase of MTX disappearance in patient No. 3 compared to 124 minutes in the nontoxic infusions, and a markedly prolonged final phase, with a half-life of 64 hours in patient No. 3 compared to 10.4 hours in the other patients.

The primary event leading to severe toxicity in this patient appeared to be the onset of renal failure during the first 24 hours of treatment, with subsequent delayed clearance of the drug, ineffective CF rescue, and severe bone marrow and gastrointestinal toxicity. The reason for renal failure is not entirely clear, but may well have been related to the failure to achieve adequate urine volume and alkalinization during the infusion, with precipitation of MTX in the renal tubules. While the initial doses of CF in the first 48 hours were unable to prevent myelosuppression, repeated administration of the antagonist beyond this period, as prompted by the clinician's awareness of delayed MTX excretion, may have prevented more prolonged and severe myelosuppression.

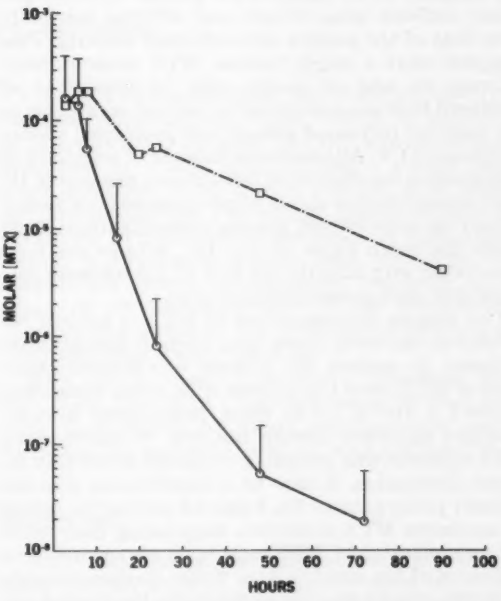

FIGURE 3.—Plasma MTX clearance curve for nontoxic infusions of 50 mg/kg (O— O). Each time-point represents the mean plasma MTX concentration from 3 infusions. Two standard deviations above the mean are noted at each time-point. The plasma MTX clearance curve of patient No. 3 (50 mg/kg, □- - -□) is also plotted for comparison.

DISCUSSION

Jaffe et al (2) have reported the use of 6-hour infusions of large doses of MTX (50-500 mg/kg) followed by CF administration in the treatment of metastatic osteogenic sarcoma and as adjuvant chemotherapy following primary surgery for localized disease (1). In the present study, the plasma and urinary pharmacokinetics of MTX, given according to the Jaffe regimen, have been determined with the aim of further understanding the relationship between pharmacokinetics and clinical toxicity.

Peak plasma levels following infusion of 50-200 mg/kg exceeded 1×10^{-4} M and were proportional to the dose administered. The peak plasma MTX concentrations were approximately 100-fold higher than peak levels achieved after conventional doses of MTX without CF rescue (9). This regimen achieves a plasma concentration far higher than conventional doses, and it is worth noting that the mechanism of action, the interaction with other agents, and the membrane transport properties of MTX in this high-concentration range have not been defined in either human or animal tumor systems.

The plasma MTX half-life of 2.06 ± 0.16 hours following the 6-hour infusion period is in agreement with the value of 2-3.4 hours reported by Pratt et al (12) and the second-phase half-life of 3.49 ± 0.55 hours following conventional doses reported by Huffman et al (9). The slower final phase of MTX clearance, usually seen between 12 and 24 hours in animals (13) and in man (9) following conventional doses, occurred in this study only after 30 hours. MTX concentration during the final phase of plasma clearance was in the range of 10^{-7} M following both high-dose MTX infusion and conventional 30-mg/m^2 iv doses. This slow "third"-phase half-life is probably responsible for the major portion of the bone marrow and gastrointestinal toxicity following conventional doses of MTX (14,15); however, in the high-dose regimen, concentrations at or below 10^{-7} M appear to have little potential for toxicity in the face of repeated administration of CF.

Huffman et al (9) noted a longer final phase half-life of 26.99 ± 4.44 hours at plasma MTX concentrations in the range of 10^{-7} M after conventional doses (30 mg/m^2 iv). The discrepancy between these values may be due either to the difference in dose schedule or the difference in assay method used. The MTX radioassay used by Huffman et al detects MTX metabolites as well as parent compound.

The plasma disappearance curves of MTX following infusion of 50 mg/kg were similar in three patients without toxic effects and differed markedly from that of the patient who exhibited toxicity. This suggests that a single plasma MTX measurement, between 24 and 48 hours, may be predictive of imminent toxicity and might be utilized as a guide to the need for increased dosage and prolonged administration of CF. Although the number of infusions in this study is insufficient to determine a predictive 48-hour concentration above which bone marrow toxicity may be experienced, plasma concentrations at or below the mean value of 2×10^{-7} M have not been associated with toxicity for any of the infusion doses (50-200 mg/kg) used in this study.

The plasma disappearance of MTX in patient No. 3 differed markedly from that seen in the nontoxic patients. In patient No. 3 there was delayed clearance of MTX from the plasma with levels remaining above 1×10^{-7} M for 16 days. Impairment in renal function apparent during the first 24 hours after MTX infusion was probably responsible for this delayed elimination. It may be of significance that the urinary pH in patient No. 3 was 5.0 during the period of maximum MTX excretion, suggesting that tubular precipitation of MTX may have played a role in the onset of his renal failure. Other studies presented in this report provide evidence for the limited solubility of MTX in acid urine, and demonstrate that urinary levels of MTX during high-dose infusion far exceed this limit. It would thus seem important to maintain adequate urine flow and alkalinization in all patients receiving high-dose MTX treatment, particularly during the first 12 hours of infusion when the highest urinary concentrations are present.

The present study and previous work (12) have provided data regarding peak plasma levels, plasma disappearance, and urine concentrations of MTX during and after high-dose 6-hour infusions. The variations in pharmacokinetics responsible for toxicity are less well defined and require further study, but delayed excretion secondary to renal failure appears to be an important mechanism. It is clear that rational planning of new regimens and safe usage of existing schedules can only be realized with the benefit of pharmacologic monitoring.

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Clinical Pharmacology of High-Dose Methotrexate (NSC-740)[1,2]

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The clinical pharmacology of methotrexate (MTX), administered in conventional oral and intravenous (iv) doses, has been studied extensively in man utilizing a variety of assay techniques (1-4). However, only a few studies (5-7) have been undertaken to investigate the pharmacology of high doses of MTX followed by delayed rescue with citrovorum factor (CF).[7] We report here results obtained through serial monitoring of serum levels of MTX in patients receiving doses of 1 g/m^2 or greater during phase I-II clinical trials. Toxicity occurred frequently in adult patients despite CF rescue and could be predicted on the basis of serum levels obtained 48-96 hours after the initiation of treatment. In addition, the presence of cytotoxic concentrations of MTX in cerebrospinal fluid (CSF) and pleural effusions was documented following high-dose systemic treatment. The benefits and limitations of serum MTX levels as a means of monitoring patients undergoing MTX/CF therapy will be presented and discussed.

METHODS

Fifty-two patients received MTX treatment either as participants in a phase I-II study of high-dose MTX/CF or in a phase II study of a regimen including vincristine, adriamycin, and MTX/CF.[8] In the first program, MTX (1-15 g/m^2) was given as a bolus or a 20-hour infusion and CF was started at 24 hours in an iv push (40 mg/m^2) followed by oral administration (25 mg/m^2) every 6 hours for 12 doses. Full details of the patients treated, the treatment program, the toxic effects, and the therapeutic responses are detailed in an accompanying communication (8). In the second program, the dose of MTX was limited to 1 g/m^2 given as a bolus and the rescue with CF was as described above. It should be noted that the patients studied were not receiving the 6-hour high-dose infusion program described by Jaffe (9). Serum, pleural fluid, and CSF were assayed for MTX using the methods described by Bertino and Fischer (10). Dihydrofolate reductase purified from *Lactobacillus casei* was a gift from Dr. R. L. Kisliuk. MTX levels in the range of 10^{-2}-10^{-8} M could be easily measured in tissue fluid. Standard curves using stock solutions of MTX were assayed with clinical specimens each day. The optical density change was within 5% in triplicate experiments. In later experiments, a radioimmunoassay was employed with comparable results and greater precision in the range of 10^{-7}-10^{-8} M. Details on the development, specificity, and use of the radioimmunoassay have been published separately (11).

MTX blood levels 1-72 hours following bolus injections and 24-72 hours following 20-hour infusions were studied by linear and quadratic regression analysis using the least-squares method (12).

RESULTS

There were ten patients (13 courses) in whom at least four serum MTX levels were taken between 1 and 72 hours following MTX administration; figure 1 shows the serum decay. The doses administered ranged from 1 to 4 g/m^2. Each line represents a best linear fit computed from these serum MTX levels. The courses that resulted in significant myelosuppression (either a white blood cell count < 2000 cells/mm^3, a platelet count < 100,000 cells/mm^3, or both) are represented by interrupted lines, and those that did not produce toxicity are represented by continuous lines. These results clearly indicate that those patients destined to have toxicity have a delayed clearance of MTX. Nineteen courses were studied by regression analysis. Of 13 bolus courses, linear regression fit the MTX levels closely in 12; curvilinear fit was as close in three instances. Of six infusion courses, either method of analysis gave equivalent results with two exceptions in which curvilinear fit was superior.

Figure 2 is a scattergram of the serum MTX levels measured in 34 patients who received MTX as a bolus injection and figure 3 is a scattergram of levels in 18 patients following 20-hour infusions. After bolus injection (dose range, 1-15 g/m^2), the serum concentration initially ranged from 10^{-2} to 10^{-4} M and then decayed with a half-life of 4.6-8.6 hours (median, 5.5 hours). Patients who were to have myelosuppression had MTX levels initially in the same range as normal, but approximately 48 hours after the bolus the "toxic" group separated from the "nontoxic" group by retaining substantially higher

[1]Supported in part by Public Health Service grant CA-06516 from the National Cancer Institute (NCI), National Institutes of Health, Department of Health, Education, and Welfare.

[2]Methotrexate: CAS reg. No. 59-05-2; glutamic acid, *N*-[*p*-[[(2,4-diamino-6-pteridinyl)methyl]methylamino]benzoyl]-.

[3]Division of Medical Oncology, Sidney Farber Cancer Center, Harvard Medical School, Boston, Mass.

[4]*Present address:* Charing Cross Hospital, London, England.

[5]We thank K. Widden for technical assistance and Y. Bishop and staff for statistical support.

[6]*Reprint requests to:* E. Frei III, MD, Sidney Farber Cancer Center, Harvard Medical School, 35 Binney St, Boston, Mass 02115.

[7]Citrovorum factor: NSC-3590; CAS reg. No. 6035-86-5; glutamic acid, *N*-[*p*-[[(2-amino-5-formyl-5,6,7,8-tetrahydro-4-hydroxy-6-pteridinyl)methyl]amino]benzoyl]-, calcium salt.

[8]Vincristine: NSC-67574; CAS reg. No. 2068-78-2; leurocristine, sulfate (1:1).

Adriamycin: NSC-123127; CAS reg. No. 23214-92-8.

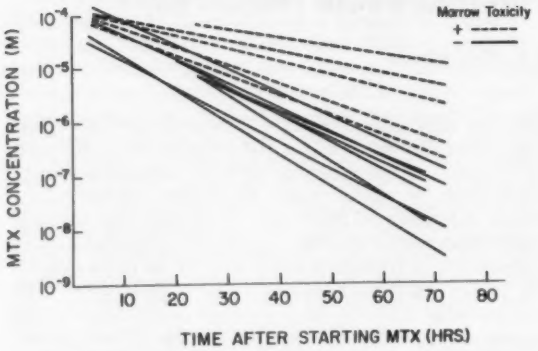

FIGURE 1.—MTX serum curves and relation to toxicity. Courses resulting in myelosuppression are represented by interrupted lines; courses without myelosuppression are represented by solid lines.

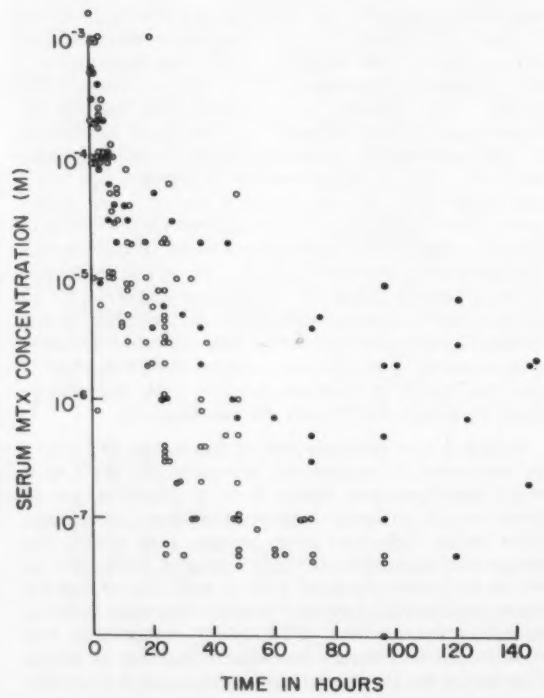

FIGURE 2.—Scattergram of serum MTX levels following iv bolus administration. Levels obtained in patients who eventually manifested toxicity are represented as closed circles; levels obtained in patients who did not manifest myelosuppression are represented as open circles.

serum MTX levels. During the infusion (1-15 g/m^2) the serum concentration of MTX ranged from 10^{-3} to 10^{-5} M, and immediately after completion it fell rapidly with a half-life of 6.4-10 hours (median, 8.1 hours). The separation of toxic and nontoxic serum MTX levels in the infusion group (fig 3) is not apparent until considerably later than in the group receiving a bolus (fig 2), and comparison of the two figures makes it clear that many patients who received infusion without toxicity had MTX levels in the toxic range of the bolus group at 48 and 72 hours. Six patients (five bolus and one infusion) who developed severe toxicity had half-lives of 10.0-25.1 hours (median, 14.2 hours).

Table 1 indicates that MTX penetrated readily into pleural fluid during the course of systemic MTX administration. In patient No. 2, the clearance of MTX from this "third space" was less rapid than in the serum; in the other patients there was a parallel decline in concentrations.

Table 2 shows that CSF-MTX levels greater than 10^{-6} M have been achieved in patients undergoing high-dose systemic therapy. The half-life in this compartment following either systemic or intrathecal therapy is 12-18 hours (13)[9] as compared to the serum half-life of 4-6 hours (noted above). This difference in half-life is reflected in the increasing CSF/serum ratio with time following high-dose systemic MTX administration (table 2).

TABLE 1.—Levels of MTX in pleural fluid following high-dose treatment

Patient No.	Time* (hrs)	MTX concentration (M)	
		Serum	Pleural fluid
1	3	3×10^{-5}	
	20	4×10^{-5}	2×10^{-5}
	44	3×10^{-7}	
	70	2×10^{-7}	
	96	3×10^{-8}	5×10^{-8}
2	24	5×10^{-7}	5×10^{-6}
	72	6×10^{-8}	3×10^{-7}
	168	1×10^{-8}	6×10^{-8}
3	120	3×10^{-6}	3×10^{-6}
4	24	3×10^{-6}	
	48	7×10^{-7}	4×10^{-7}
	72	3×10^{-7}	
5	12	9×10^{-6}	9×10^{-6}
6	6	1×10^{-4}	5×10^{-5}

*Time after giving bolus or initiating 20-hr infusion.

Table 3 shows the simultaneous serum and CSF levels of MTX in patient No. 11 who received 5.4 g/m^2 of MTX by bolus or 20-hour infusion as treatment for lymphomatous meningitis. This patient responded well to treatment with a rapid but temporary clearing of CSF blast cells.

[9]Tattersall MHN, et al. Unpublished data.

TABLE 2.—Levels of MTX in CSF following high-dose treatment

Patient No.	Time (hrs)	Mode of administration*	MTX concentration (M)		Ratio of CSF/serum
			Serum	CSF	
7	5	I	1×10^{-4}	8×10^{-6}	0.08
8	15	B	5×10^{-5}	8×10^{-7}	0.016
9	20	B	5×10^{-5}	4×10^{-6}	0.08
10	20	I	2×10^{-5}	4×10^{-6}	0.05
11	22	I	3×10^{-5}	6×10^{-6}	0.05
11	22	B	2×10^{-6}	2×10^{-6}	1.0
7	48	I	3×10^{-8}	7×10^{-8}	2.3
12	96	I	5×10^{-8}	9×10^{-8}	1.4
11	96	I	6×10^{-8}	4.6×10^{-8}	0.77

*B = MTX given as bolus; I = MTX given in 20-hr infusion.

TABLE 3.—Treatment of meningeal lymphoma (patient No. 11) with high-dose systemic MTX

Treatment*	Time† (hrs)	MTX concentration (M)		Cell count (blasts/mm³)	Cytocentrifuge morphology
		Serum	CSF		
Before treatment	—	—	—	100	All blasts
Infusion	22	3.0×10^{-6}	6.0×10^{-6}	80	50% blasts
	96	6.0×10^{-8}	4.6×10^{-8}	0	No blasts
Infusion	24	2.2×10^{-5}	3.6×10^{-6}	15	Rare blasts
Infusion	23	2.3×10^{-5}	2.7×10^{-6}	8	No blasts
Bolus	22	2.1×10^{-6}	2.1×10^{-6}	4	No blasts
Bolus	23	1.5×10^{-6}	1.2×10^{-6}	6	No blasts
Bolus	23	1.7×10^{-6}	1.0×10^{-6}	3	Rare blasts
Bolus	22	4.6×10^{-6}	1.7×10^{-6}	5	Rare blasts

*5.4 g/m² by 20-hr infusion or bolus injection.
†Time after initiation of MTX treatment.

DISCUSSION

In an early study of MTX pharmacology, Henderson et al (2) administered conventional doses of MTX (up to 10 mg/kg) with tritiated MTX and observed exponential plasma decay with a mean half-life of 134 minutes in the first 8 hours. With MTX doses of 30 mg/m² Huffman et al (3) defined a triphasic plasma disappearance curve with half-lives of 0.75, 3.5, and 27 hours when levels of MTX were determined over 60 hours of observation. Pratt et al (7), using doses comparable to those employed in this study, defined a mean half-life of 2.6 hours in patients receiving 6-hour infusions and observed for 30 hours.

The patients studied here were observed over longer periods of time with a major goal being to correlate MTX levels with potential therapeutic effects or the development of toxicity. Serum levels obtained from 1 to 72 hours after treatment were studied by regression analysis. Decay over this time period was exponential in most nontoxic patients with a median half-life of 7.2 hours. This value probably represents a composite of the two longer half-lives described above which were determined with multiple early and late sampling and through more sophisticated pharmacokinetic modeling. Persistence of low levels of MTX ($2\text{-}5 \times 10^{-8}$ M) in serum for periods beyond 72 hours was observed in nontoxic patients. This is compatible with the slow displacement of bound MTX from tissues which is believed to account for the final phase of plasma disappearance. It is also possible that metabolites of MTX may be present at these later time points although their concentration would have to be greater than 10^{-7} M for detection by the radioimmunoassay.

Patients destined to have myelotoxicity could be identified by their tendency to retain higher serum

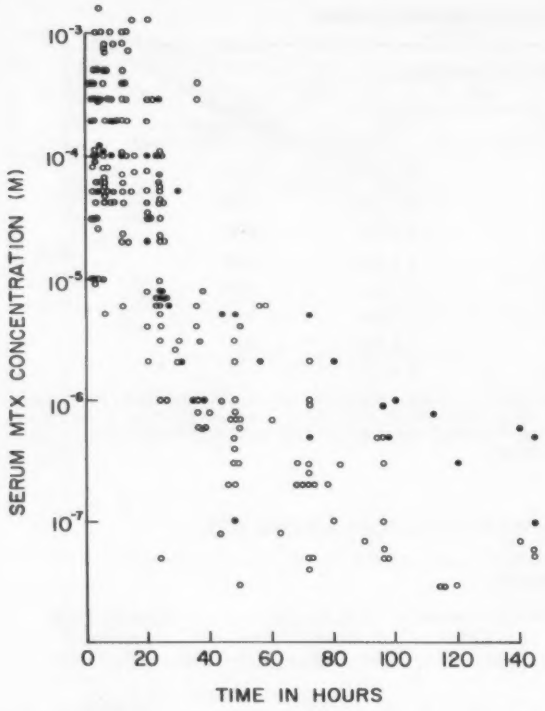

FIGURE 3.—Scattergram of serum MTX levels following 20-hr iv infusion. Symbols same as in fig 2.

MTX levels than nontoxic patients at varying times following treatment initiation. All but two nontoxic patients receiving bolus injections had serum values of less than 5×10^{-7} M at 48 hours and all nontoxic patients had values at or below 1×10^{-7} M at 72 hours. Thus, the fixed dose and schedule of CF employed in this study will prevent MTX toxicity in patients whose clearance is adequate to reduce the serum MTX concentration to those levels. In contrast, patients receiving infusions could retain levels of up to 5×10^{-7} M for 96 hours without exhibiting toxicity. The reason for this discrepancy is unknown and probably complex, but emphasizes differences which may arise on the basis of the mode of drug administration.

The penetration of MTX into pleural fluid was documented in several patients. It is clear that in the presence of pleural fluid the volume of distribution of MTX will increase, and it is possible that under certain conditions, it may act as a depot resulting in a longer serum half-life and a potential for toxicity. Many of the patients who had toxicity had pleural effusions or other "third space" problems in addition to renal impairment, but the contribution of the "third space" to toxicity could not be isolated.

Therapeutic concentrations of MTX may appear in CSF, and beneficial results of treatment in the patient presented here and in one patient elsewhere (14) suggest the need for further study of this modality of treatment. MTX was cleared more slowly from this compartment than from serum, a finding reflected in the gradually increasing ratio of CSF/serum MTX concentrations.

It is important to note that an individual MTX level was not of definitive predictive value for the development of toxicity until 2-4 days after the initiation of therapy. This conclusion probably resulted because the data obtained from many dosage levels were pooled according to the mode of administration. If a single dose level is employed, if the mode of administration is defined, and if the rescue program is specified, it should be possible to detect delayed MTX clearance much earlier and relate it to toxicity. Such patients could be identified by carefully observing renal function over the first 24-48 hours of therapy. Prior studies (15) have suggested that no more than 48 hours of continuous MTX exposure may be adequately rescued by CF. If meaningful alterations in the dose and schedule of CF as well as methods for degrading MTX (16) are to be employed with some hope of preventing severe toxicity to bone marrow and intestinal epithelium, early recognition of delayed MTX clearance is essential.

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Toxicity of High-Dose Methotrexate (NSC-740) and Citrovorum Factor (NSC-3590) in Osteogenic Sarcoma[1,2]

Norman Jaffe and Demetrius Traggis[3,4]

SUMMARY

Three regimens of high-dose methotrexate (MTX) followed by citrovorum factor (CF) were utilized in the management of osteogenic sarcoma. Treatment comprised vincristine (2 mg/m^2; maximum dose, 2 mg) followed ½ hour later by MTX (1500 mg/m^2 escalated to 7500 mg/m^2 in 2-3 weeks) administered intravenously (iv) over 6 hours. Two hours later, CF (5-15 mg) was administered by the iv and oral route for 72 hours. The most recent regimen incorporated adriamycin (75 mg/m^2) into the treatment protocol. Five hundred and forty-two courses of high-dose MTX were administered in 41 patients. Prerequisites for treatment included a normal creatinine clearance and hemogram. Side effects comprised stomatitis, myelosuppression, nausea and vomiting, convulsions, and abnormalities in renal and liver function studies. There were two drug-related deaths. Toxicity proved to be erratic and unpredictable, but analysis of its development suggested that an increased frequency was associated with dehydration, infection, fever, and a "third space." It is suggested that the safety of MTX/CF regimens may be improved by frequent pharmacologic monitoring of serum MTX levels and ensuring adequate hydration to promote renal excretion of the drug.

[Cancer Chemother Rep Part 3, vol 6:31-36, 1975]

The therapeutic effect of high-dose methotrexate and citrovorum factor rescue (MTX/CF) in osteogenic sarcoma has been demonstrated in a number of communications (1-6). Initial reports from the Sidney Farber Cancer Center emphasized the erratic and unpredictable nature of toxicity, stressed the prerequisites for treatment, and recommended that the regimen be employed under expert medical supervision where resources such as blood products and pharmacologic testing procedures were available. This communication will examine the toxicity in greater detail.

MODIFICATIONS OF MTX/CF PROGRAM

During the past 2 ½ years, the MTX/CF regimen has undergone modification designed to improve its effectiveness and safety. These modifications are as follows: (*a*) Pretreatment with vincristine[5] has been introduced based on experimental evidence that it promotes MTX uptake by tumor cells (7). (*b*) Adriamycin[6] has been incorporated into the therapeutic regimen and the intramuscular form of CF has been replaced by an intravenous (iv) form during the first 24 hours followed by an oral administration for the ensuing 48 hours. Three treatment regimens have been studied and are outlined in the following sections.

Regimen 1: Vincristine Plus MTX/CF

This regimen was administered at 3-week intervals (fig 1), and was similar to the treatment scheme adopted in the initial investigations (1,2). Treatment starts with vincristine (2 mg/m^2; maximum dose, 2 mg) administered iv with a scalp tourniquet maintained at 120 mm of mercury for 3-5 minutes. One-half hour later, MTX dissolved in 600 cc of 5% dextrose and water is administered by iv infusion over 6 hours. The initial dose is 1500 mg/m^2 and is escalated to 3000 and 6000 mg/m^2 at 2-week intervals. Three

	VINCRISTINE	2 mg/m^2		
	HALF HOUR LATER	MTX		
	EIGHT HOURS LATER	CF		
INTERVAL BETWEEN DOSES	MTX (mg/m^2)	CITROVORUM FACTOR (mg)		
		<5	10	>10 (m^2)
2 WEEKS	1500, 3000, 6000	5	10	15
3 WEEKS	7500			
ADMINISTRATION	6 HOURS	I.V.I. q3h x 24 HRS. Followed by P.O. q6h x 48 HRS.+		

+ First oral dose commences 3 hrs. after last I.V. dose.

FIGURE 1.—Regimen 1 doses and schedule: vincristine plus MTX/CF in patients with osteogenic sarcoma and no clinical evidence of metastases.

[1]Supported in part by grant CA-06516-12 (National Cancer Institute) and grant PR-05526-12 (Division of Research Facilities and Resources), National Institutes of Health, Department of Health, Education, and Welfare.

[2]Methotrexate: CAS reg. No. 59-05-2; glutamic acid, *N*-{p-[[(2,4-diamino-6-pteridinyl)methyl]methylamino]benzoyl}-; MTX.

Citrovorum factor: CAS reg. No. 6035-86-5; glutamic acid, *N*-{p-[[(2-amino-5-formyl-5,6,7,8-tetrahydro-4-hydroxy-6-pteridinyl)-methyl]amino]benzoyl}-, calcium salt.

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[4]*Reprint requests to:* Norman Jaffe, MD, Sidney Farber Cancer Center, Harvard Medical School, 35 Binney St, Boston, Mass 02115.

[5]Vincristine: NSC-67574; CAS reg. No. 2068-78-2; leurocristine, sulfate (1:1).

[6]Adriamycin: NSC-123127; CAS reg. No. 23214-92-8.

weeks later, the dose of MTX is increased to 7500 mg/m² and is maintained at this level for 2 years.

Two hours after the termination of the MTX infusion, CF (5-15 mg) is administered iv at 3-hour intervals for eight doses. The dose is dependent on the surface area of the patient and the amount of MTX administered. Three hours after the last iv dose, oral CF tablets in the same dose are prescribed. They are administered at 6-hour intervals for eight doses provided there is no nausea and vomiting. If nausea and vomiting supervene, iv treatment with CF at 3-hour intervals is resumed. The total duration of CF rescue is thus 72 hours. Entry into this study was terminated in June 1974.

Regimen 2: Vincristine Plus MTX/CF Plus Adriamycin

This treatment is similar to regimen 1. However, the initial dose of MTX is 3000 mg/m², and after the 6000-mg/m² dose has been attained adriamycin (75 mg/m²) is introduced (fig 2). It is administered as a pulse dose 7 days after the start of each vincristine plus MTX/CF course. Since the nadir of myelosuppression from adriamycin generally occurs within 10-14 days, the next course of vincristine plus MTX/CF is administered on Day 28. Further doses of the three agents are administered at monthly intervals with adriamycin following 7 days after each course until a total cumulative dose of 450 mg/m² of adriamycin has been attained. Thereafter, vincristine plus MTX/CF treatments continue at 3-week intervals until a 2-year course of chemotherapy has been administered. This study was activated in June 1974.

Regimen 3: Weekly Vincristine Plus MTX/CF Treatment

Investigations are in progress to determine the efficacy and safety of administering weekly courses of vincristine plus MTX/CF to patients with pulmonary metastases resistant to previous adriamycin therapy or other vincristine plus MTX/CF schedules. The dose escalations of vincristine plus MTX/CF are identical to those described earlier, with therapy administered at weekly intervals for a maximum of 6 weeks. Further treatment is determined by the patient's response.

TREATMENT-RELATED STUDIES AND PROCEDURES

Pretreatment evaluation for vincristine plus MTX/CF includes a complete blood count, liver function studies, creatinine clearance, and estimations of serum calcium, phosphorus, and magnesium. A complete blood count and urinalysis are obtained daily during treatment and liver function studies and serum MTX levels are done at the end of each course. Treatment is withheld in the presence of an abnormal creatinine clearance, clinical jaundice, severe infection, mucositis, myelosuppression (leukocyte count < 2000 cells/mm³ and platelet count < 100,000 cells/mm³), and dehydration. Throughout the MTX/CF treatment period, a continuous infusion of dextrose and water in 0.2% N saline is maintained to ensure adequate hydration. Liver function studies and hemograms are repeated at weekly intervals.

With respect to adriamycin, an electrocardiogram is obtained at the start of treatment and at the beginning of the third and last dose. Complete blood counts and liver function studies are obtained before each dose. The drug is withheld if there is any evidence of a cardiac abnormality, and if severe myelosuppression is encountered the next dose is reduced according to the guidelines outlined in table 1.

General supportive measures for vincristine plus MTX/CF plus adriamycin include platelet transfusions for platelet counts below 25,000 cells/mm³, the empirical use of antibiotics for temperatures above 102°F, and white blood cell (wbc) transfusions if infection is documented and the polymorphonuclear leukocyte count is below 500 cells/mm³. Prophylactic mouthwashes with Cepacol and hydrogen peroxide are utilized continually during vincristine plus MTX/CF treatment.

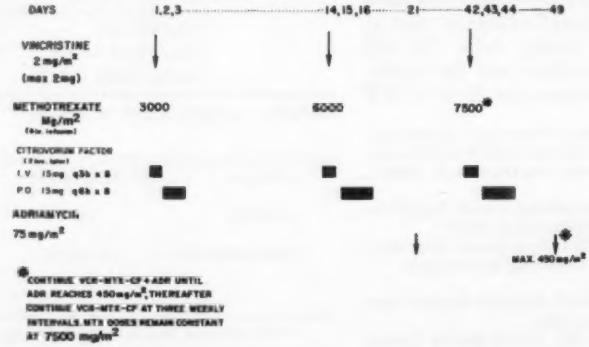

FIGURE 2.—Regimen 2 doses and schedule: vincristine plus MTX/CF plus adriamycin in patients with osteogenic sarcoma and no clinical evidence of metastases.

TABLE 1.—Dose adjustments for adriamycin in regimen 2 of MTX/CF, determined by hematologic toxicity in previous course*

Percent of dose given	If wbc or granulocyte nadir was— Wbc (cells/mm³)	Granulocytes (cells/mm³)	and/or	If platelet nadir was— (cells/mm³)
100 (75 mg/m²)	1000-2000	300-1200		50,000-100,000
75	500-1000	200-300		25,000- 50,000
50	< 500	<200		<25,000

*If previous dose had been sharply reduced because of myelosuppression, upward modification to a maximum of 75 mg/m² could proceed by larger increments.

EVALUATION OF TOXICITY

The toxicity of vincristine plus MTX/CF was analyzed by reviewing the records of patients treated with the different regimens from February 1972 to December 1974 (table 2). The earlier experiences were analyzed with regimen 1, which involved minimal departures from the original program.

Five hundred and forty-two courses of vincristine plus MTX/CF were administered to 41 patients as follows: regimen 1, 475 courses; regimen 2, 35 courses; and regimen 3, 32 courses (table 3). Thirty-two patients received regimen 1, seven patients received regimen 2, and four patients (including two originally treated with regimen 1) received regimen 3. Two patients treated in the early experience with regimen 1 died of possible drug-related causes: the first of myelosuppression and superimposed *Clostridium perfringens* infection, and the second of a possible vasovagal attack during nasopharyngeal suction for stomatitis.

Stomatitis

Forty-nine courses (9%) were associated with stomatitis. This varied from occasional areas of ulceration of the lips and buccal mucosa to severe nasopharyngeal mucositis, denudation of the mucosa, and production of thick tenacious viscid sputum. Most of the latter cases were associated with severe myelosuppression and occasional episodes of jaundice.

Myelosuppression

Myelosuppression, defined as a wbc below 1500 cells/mm³ or a platelet count below 100,000 cells/mm³, was encountered in 29 courses (5%). It generally occurred 4-5 days after cessation of CF treatment. The lowest wbc and platelet counts recorded were 100 and 5000 cells/mm³ respectively. Several cases were associated with fever around 102°F, although evidence of sepsis was not detected except in one patient who died of *Clostridium welch'ii* infection, as indicated earlier. Wbc and platelet support and antibiotics were prescribed when indicated and fever generally abated within 7-10 days.

TABLE 2.—Analysis of toxicity from 542 courses of vincristine plus MTX/CF

Toxicity	No. of courses	Percent of total courses
Stomatitis	49	9
Myelosuppression	29	5
Liver function abnormalities (results from 250 courses)		
Elevation of serum transaminase	152	28
Pretreatment elevation of serum transaminase	55	10
Elevation of serum bilirubin	10	2
Nausea and vomiting	210	39
Renal abnormalities	6	<1
Dermatologic abnormalities	6	<1
Convulsions	3	<1

TABLE 3.—Analysis of regimens of vincristine plus MTX/CF with or without adriamycin

Regimen	No. of courses	No. of patients
1	475	32
2	35	7
3	32	2 (plus 2 from regimen 1)
Total	542	41

Liver Function Abnormalities

Two hundred and fifty courses of treatment provided information on the serum alkaline phosphatase, transaminase, bilirubin, and protein levels. Of these, elevations in the serum transaminase levels were recorded in 152, and elevations in serum bilirubin were recorded in ten. Serum oxaloacetic transaminase levels were mildly to moderately abnormal before treatment in 55 patients. In the majority of patients, these levels returned to normal before the next scheduled course of therapy, indicating an absence of progressive liver dysfunction. The tests of serum alkaline phosphatase and proteins did not reveal any consistent or major abnormalities after therapy.

Autopsy studies were available for four patients. They had received three to ten courses of treatment and abnormal liver function tests were detected in four of these courses. The patients had survived 6-18 months after the start of vincristine plus MTX/CF treatment and there was no evidence of cirrhosis, fibrosis, or hepatocellular disease.

Renal Abnormalities

Six patients exhibited evidence of severe renal abnormalities. These occurred between the third and 11th course of treatment. Manifestations of renal dysfunction were oliguria, anuria, and electrolyte disturbances accompanied by elevations of the serum creatinine and the blood urea nitrogen. Hydropic epithelial cells were detected in the urine of one patient who had a fixed specific gravity followed by diuresis and a return to normal renal function. The two patients who died of drug-related causes also had oliguria. A fourth patient had dehydration, elevated levels of circulating serum MTX, and anuria. Treatment for acute renal failure was initiated and was followed several days later by a return to normal renal function. A fifth patient had oliguria, severe electrolyte disturbances, and persistent hypertension after the sixth course of treatment. Restoration of normal renal function and blood pressure was achieved with conservative medical management. A similar therapeutic approach resulted in resumption of normal renal function in the sixth patient who had temporary oliguria. Extension of CF rescue was provided in all instances for treatment of myelosuppression and stomatitis, and reinstatement of vincristine plus MTX/CF has been possible in all four surviving patients.

Dermatologic Abnormalities

Rashes, exacerbation of acneiform eruptions, superficial sloughing in decubitus parts of the body, and erythema and desquamation confined to portals of irradiation were observed in six patients. These resolved over several weeks and were progressively less reproducible with repeated treatments. Total alopecia occurred in all patients receiving adriamycin; partial alopecia which occasionally appeared with vincristine plus MTX/CF generally improved as treatment continued.

Nausea, Vomiting, and Dehydration

Approximately 40% of the courses were associated with nausea and vomiting. The majority of these complications were usually mild and were well controlled or prevented by antiemetics. However, occasionally the symptomatology was complicated by dehydration and other manifestations of toxicity. Recognition of the dehydration phenomenon and correction of plasma volumes appeared to account for a decreasing incidence of toxicity as experience accumulated.

Convulsions

Three patients had convulsions: one following vincristine plus MTX/CF administered in the immediate postoperative period, another with the fourth treatment, and a third with the third weekly treatment. The etiology of the convulsions was not determined. All patients subsequently tolerated additional treatments without untoward effects although further vincristine was omitted in one of these patients.

DISCUSSION

These results demonstrate that vincristine plus MTX/CF was well tolerated in over 85% of the courses. However, the regimens were not entirely free of side effects. Most of the complications were encountered with regimen 1 which has been extensively used during the past 3½ years; experiences with the other regimens are presently insufficient to determine if the recent modifications will result in any change.

An increased incidence of toxicity was associated with dehydration generally due to nausea and vomiting. The latter usually occurred during the early period of the MTX infusion and occasionally accompanied an undetected infection. Dehydration probably enhances the potential for toxicity by increasing

the serum MTX concentration and reducing renal excretion. Vincristine plus MTX/CF should consequently not be administered to any patient who is dehydrated, and excessive vomiting during treatment should be counteracted with vigorous iv therapy. CF rescue should also be extended beyond 72 hours, as dictated by the results of the serum MTX level under these circumstances.

MTX is excreted by the kidney and a delay in renal excretion by failing kidneys may accentuate toxicity (8). Creatinine clearance tests were, therefore, utilized as an index of renal function. The results were consistently normal in all patients before each treatment but did not preclude the development of toxicity. Occasionally, transient abnormalities in creatinine clearance tests could not be substantiated on repeat testing and were possibly due to inadequate collections of urine. These tests were performed just prior to therapy. More frequent determinations, particularly during or immediately after therapy, have revealed 25% elevations in the serum creatinine of patients receiving alternate high-dose MTX therapy (9). Such minor aberrations may possibly also occur in the osteogenic sarcoma regimens and contribute to the development of toxicity. Studies addressed to this problem have recently been initiated.

In addition to being excreted by the kidneys, MTX is a known nephrotoxic agent, causing functional and morphologic changes in the epithelium of the convoluted tubules (10). Its precipitation in the distal tubule may result in tubular dilatation, oliguria, and anuria (9). These complications, however, are reversible. Vigorous treatment for renal failure should be implemented and CF should be administered for elevated serum MTX levels. Investigations are currently in progress to determine if reduction in serum MTX levels can be achieved through exchange transfusions or hemodialysis. With complete recovery, re-instatement of vincristine plus MTX/CF treatment is possible. Preliminary studies would also suggest that alkalinization of the urine, permitting increased excretion of MTX, may prevent the development of renal toxicity (9).

The data did not reveal evidence of permanent liver damage. Transient disturbances in serum enzyme levels following treatment generally resolved in 1-2 weeks. Similar observations have been reported by Djerassi et al (11). Consequently, slight abnormalities in hepatic enzyme studies were not considered a contraindication for treatment. However, treatment was temporarily interrupted if an elevated serum bilirubin level was detected. The latter was not reproducible with repeated courses.

Myelosuppression accompanied all severe complications. This side effect occasionally recurred in subsequent courses, but no patient experienced more than three episodes. Approximately 10% of the myelosuppressive complications were associated with polymorphonuclear leukocyte counts below 1000 cells/mm^3. The nadir of myelosuppression generally occurred by the tenth to the 14th day with the hemogram returning to normal 10 days later. The latter was usually accompanied by an overshoot of the myelosuppressed elements.

Mucositis produced mild to severe degrees of discomfort. The majority of patients were treated on an ambulatory basis and achieved relief with the use of xylocaine and Cepacol mouthwashes. Several patients, however, required iv therapy to improve hydration. Occasionally, thick tenacious viscid sputum necessitated hospitalization for nasopharyngeal suction and supportive measures. Mucositis also did not recur more than three times in any one patient.

Patients with pleural or peritoneal effusions (a "third space") have demonstrated a prolonged plasma MTX half-life due to a delay in the excretion of the drug from the extravasated areas (12). A similar problem with sequestration of MTX into edematous tissues (a possible "third space") during the postoperative period was possibly responsible for toxicity in one patient. Treatment was initiated several hours after a hemipelvectomy and resulted in mucositis and myelosuppression. Moderate edema at the operative site and an elevated serum MTX level were noted several days later. This patient subsequently tolerated higher doses of MTX without untoward effects. The advisability of administering MTX in the immediate postoperative period is currently under re-evaluation. Consideration is also being given to extending the period of CF rescue to patients with potential "third space" problems.

Despite the increasing experience with the vincristine plus MTX/CF regimens, toxicity still proved erratic. There did not appear to be any relationship to dose, schedule, or the number of previous courses. Several episodes of toxicity occurred with the first treatment but did not recur subsequently. Treatment in these patients was administered with appropriate attention to renal function, hydration, and investigation of infection, fever, and a "third space." A review of the MTX level in a number of these patients would suggest that concentrations in excess of 2×10^{-7} M at 72 hours were associated with these complications. An explanation for this finding was not immediately apparent and further studies are warranted. However, the results provide a basis for recommending extension of the CF rescue and continued efforts at maintaining hydration to ensure MTX excretion. These experiences further suggest that until more information is available, the safety of the vincristine plus MTX/CF regimens may be improved by frequent pharmacologic monitoring of serum MTX concentrations.

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Improvement in the Therapeutic Index of Methotrexate (NSC-740) by L-Asparaginase (NSC-109229)[1,2]

Robert L. Capizzi[3,4]

Methotrexate (MTX) has been available for clinical use for 20 years and its therapeutic spectrum continues to be explored. Studies in tumor-bearing animals have indicated that remission duration is a distinct function of dose and that "cures" are possible with the use of MTX alone. However, in order to achieve this effect, one must often pay a very significant price in terms of drug-induced morbidity and mortality.

A significant improvement in the therapeutic index of MTX can be achieved in animals (1) and man (2-5) by administering the antidote, citrovorum factor (CF),[5] following MTX treatment. The improved therapeutic results of these programs are probably the result of selective "rescue" of normal cells over the tumor cells. It has also been shown that therapeutic results may frequently be improved by using higher doses of MTX, usually with CF "rescue," and/or altering the schedule of drug administration (6). Thus, these studies have emphasized that "absolute resistance" to MTX may not be as frequent as "relative resistance" which may be a function of schedule of drug administration and dose.

The initial demonstration of schedule dependency between MTX and L-asparaginase with regard to therapeutic response (7,8) prompted further laboratory investigation of MTX toxicity with subsequent application in clinical trials (9). These observations serve as the basis for this presentation.

METHODS AND RESULTS

Studies of schedule dependency were performed with the murine L5178Y lymphoblast cell line which is sensitive to both MTX and L-asparaginase. A suspension of 10^6 cells in saline was inoculated intraperitoneally (ip) into male BDF_1 mice[6] weighing 20-25 g. All therapy was begun 3 days after tumor implantation and was administered by the ip route. As shown in figure 1, therapy consisted of saline, L-asparaginase alone at 500 IU/kg, MTX alone at 75 mg/kg given twice at 6-hour intervals, or a combination consisting of L-asparaginase followed by MTX 24 hours later or MTX followed by L-asparaginase 24 hours later. Four courses of therapy were administered at weekly intervals. The rationale which led to the design of this regimen has been previously described (8). The mice treated with L-asparaginase or MTX alone survived significantly longer than the saline-treated mice (30.8, 25.9, and 15.9 days respectively). Although both regimens employing the combination produced approximately additive results, the regimen where MTX was administered prior to L-asparaginase produced 55% 90-day survivors, whereas the converse regimen resulted in no long-term survivors. Rechallenge of the cured mice with 10^6 cells produced uniform tumor "takes" with survival times comparable to the saline-treated group, indicating that the mice were neither innately resistant to the tumor nor did they develop immunologic resistance as a result of surviving the original tumor transplant.

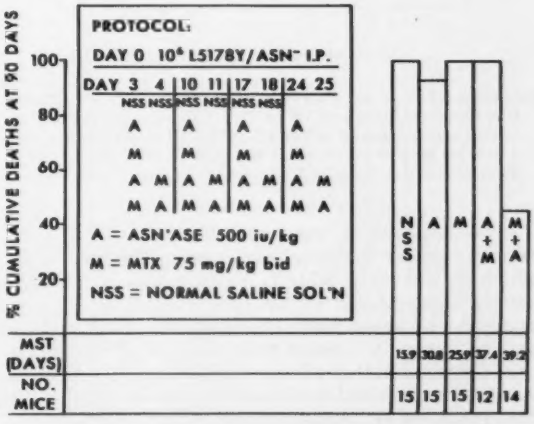

FIGURE 1.—Effect of sequence of drug administration on therapeutic efficacy. Leukemic mice were treated according to the protocol shown in the insert. MST = mean survival time; ASN'ASE = L-asparaginase. [Reprinted with permission from ref 8.]

In observing these mice it appeared that the combined regimens were less toxic than MTX alone, and consequently the effect of L-asparaginase on MTX toxicity was explored. Weight loss in treated mice was used as an index of drug toxicity. As shown in figure 2, leukemic mice treated with saline gained weight as the ascites fluid accumulated. The rate of weight increase in the L-asparaginase-treated group was slightly less than in controls, perhaps due to the

[1]Supported in part by grants IN-31-M-3 and C1-101 from the American Cancer Society.

[2]Methotrexate: CAS reg. No. 59-05-2; glutamic acid, *N*-[p-[[(2,4-diamino-6-pteridinyl)methyl]methylamino]benzoyl]-; MTX.
L-Asparaginase: CAS reg. No. 9015-68-3; Asn'ase.

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[5]Citrovorum factor: NSC-3590; CAS reg. No. 6035-86-5; glutamic acid, *N*-[p-[[(2-amino-5-formyl-5,6,7,8-tetrahydro-4-hydroxy-6-pteridinyl) methyl]amino]benzoyl]-, calcium salt; leucovorin; *N*-formyltetrahydrofolate.

[6]Purchased from Jackson Laboratory, Bar Harbor, Me.

delay in ascitic fluid accumulation secondary to the therapeutic effect of the L-asparaginase. In contrast, a single dose of MTX at 100 mg/kg caused a 10% weight loss which was all but abolished by treating the mice with L-asparaginase 3 hours before the MTX. Reversing the sequence of the drugs, ie, administering MTX 3 hours before L-asparaginase, produced neither gain nor loss of weight. The interval of 3 hours between drugs was based on studies indicating maximal biochemical interference between L-asparaginase and MTX at this time (10). During the period of observation, these mice appeared healthy with normal skin turgor and no evidence of ascitic fluid accumulation.

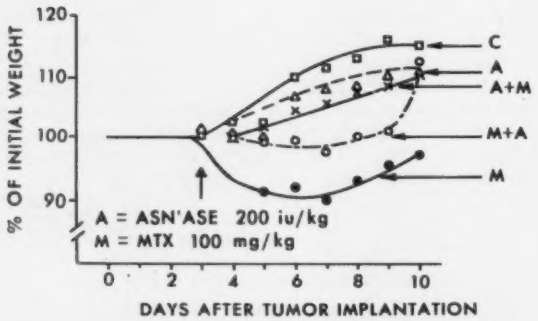

FIGURE 2.—Effect of sequence of drug administration on toxicity. Mice were inoculated with 10^6 L5178Y/ASN- cells and were treated with a single dose of saline (C), MTX (M), L-asparaginase (A), A + M, or M + A on the third day after tumor implantation. [Reprinted with permission from ref 8.]

Investigations of the toxic effects of MTX were carried further in nontumorous mice, the results of which are shown in table 1. A single dose of MTX at 500 mg/kg produced an average weight loss of 5 g and killed all seven mice. Preceding the MTX with L-asparaginase by 3 hours protected six of the seven mice from the lethal effect of MTX, and the weight loss in the combined treatment group was similar to that produced by L-asparaginase alone.

TABLE 1.—Effect of L-asparaginase pretreatment on MTX toxicity in normal BDF_1 mice

Treatment	Weight change on Day 7 (g)	No. of animals dead/ No. treated
Saline	+0.9	0/7
L-Asparaginase (500 IU/kg)	−0.5	0/7
MTX (500 mg/kg)	−4.9	7/7
L-Asparaginase* + MTX	−0.7	1/7

*Given 3 hrs before MTX.

Histologic examination of the small intestinal mucosa of the treated mice is shown in figure 3. In this experiment mice were killed 3 days after drug treatment, and a section of the ileum was placed in formalin solution, sectioned, and stained with hematoxylin and eosin. Whereas MTX at 500 mg/kg produced a complete denudation of the intestinal villi as seen in both the high- and low-power views, when L-asparaginase was administered prior to MTX the villous pattern was similar to that seen with L-asparaginase or saline.

A similar protective effect against MTX toxicity is produced in humans treated with the combination of MTX and L-asparaginase. The biochemical rationale for the combination has been presented (8,9). An example of patient tolerance to MTX as influenced by L-asparaginase is shown in figure 4. The patient is a 32-year-old woman with acute lymphoblastic leukemia; her hematologic response over a 130-day period is shown in this figure. She had previously relapsed after receiving a combination of drugs which included MTX, and she had shown signs of MTX toxicity at a dose of 100-130 mg/kg. The dose sequence and interval were based on biochemical studies performed with the patient's blast cells (8). Therapy was begun with a single dose of L-asparaginase (500 IU/kg) administered on Day 0. Ten days later she received a total dose of 120 mg of MTX which was followed by L-asparaginase 24 hours later. This 2-day course of therapy was then repeated at 10-day intervals. Each subsequent dose of MTX was always followed by 500 IU/kg of L-asparaginase at 24 hours. The dose of MTX was increased by 25%-50% with each course. Ten days after a second dose of 1.2 g of MTX on Day 90, she experienced slight erythema of the oral mucosa and a transient fall in granulocytes. The dose of MTX was subsequently lowered to 900 mg and was continued at that level. Therapy before and after this occasion was consistently associated with a platelet count in excess of 150,000 cells/mm^3 and a granulocyte count in excess of 1000 cells/mm^3. At no time did she experience other oral or gastrointestinal toxicity.

The selective cytotoxic effect of the MTX/L-asparaginase combination in a series of six patients with ALL is shown in figure 5. At the start of therapy all patients were in florid relapse of their disease and had previously been treated with multiple-drug regimens which included MTX. They received an initial single intravenous (iv) dose of L-asparaginase (500 IU/kg), and 10 days later they received a single iv injection of MTX followed 24 hours later by a single iv injection of L-asparaginase. This 2-day sequential treatment was then repeated at 9-10-day intervals (9,11). The hematologic tolerance to MTX throughout nine courses of therapy (90 days) is shown. Despite the fairly rapid escalation of the dose of MTX, from an average of 60 mg/m^2 to an average of 400 mg/m^2, the absolute number of granulocytes and platelets in the peripheral blood remained reasonably stable at approximately 2000 and 200,000 cells/mm^3 respectively. The average maximally tolerated

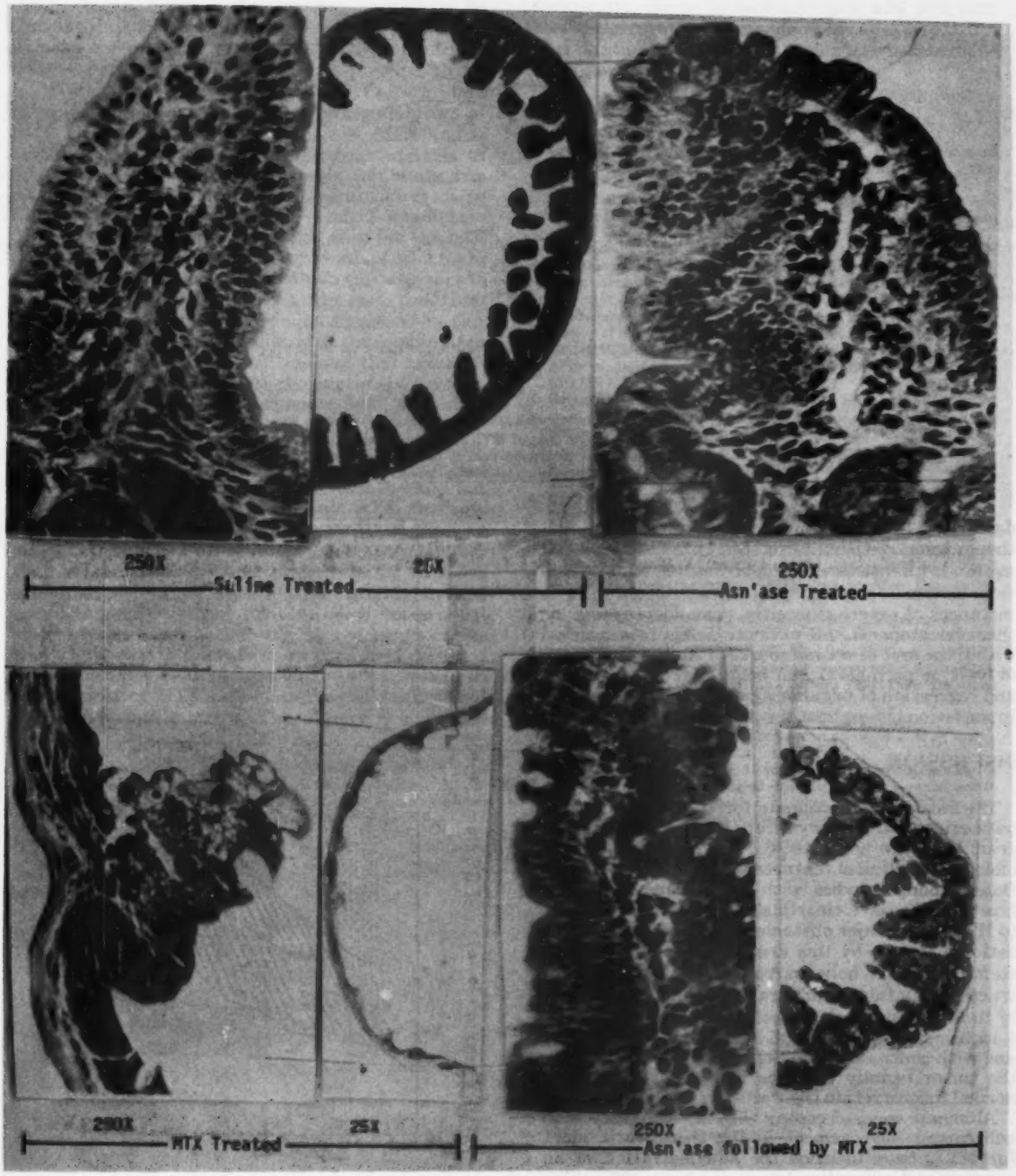

FIGURE 3.—Normal BDF_1 mice were treated as indicated (MTX, 500 mg/kg; L-asparaginase [Asn'ase], 500 IU/kg). In the combined treatment group, L-asparaginase preceded MTX by 3 hours.

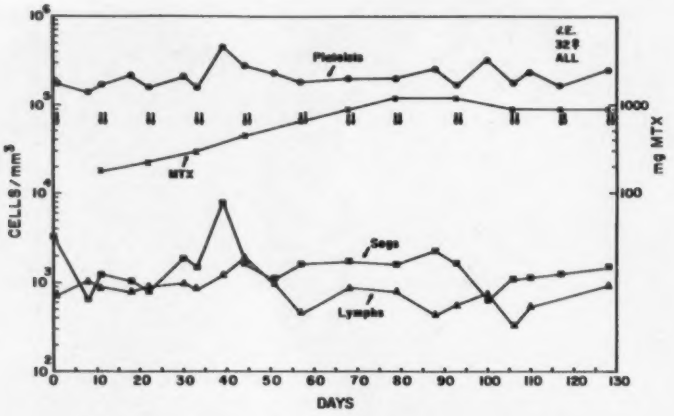

FIGURE 4.—A 32-year-old white woman with acute lymphoblastic leukemia was treated at 10-day intervals with a single dose of MTX followed 24 hours later by a single dose of L-asparaginase. Each treatment course is indicated by ↑ ↑. Segs and lymphs = absolute number of granulocytes and lymphocytes respectively per mm³ of peripheral blood.

dose of MTX was approximately 400 mg/m², although some patients were able to tolerate 600-800 mg/m². In all instances the dose-limiting toxic effects were lassitude, nausea, and vomiting. There were no instances of severe mucositis, granulocytopenia, or thrombocytopenia. All patients achieved remission within the first or second course of therapy with rapid lysis of peripheral and bone marrow blast cells and regression of organomegaly, and they remained in remission throughout the period of observation.

DISCUSSION

The biochemical rationale for the apparent optimal sequence and interval for the drugs in this program has been presented (9, 11). However, certain biologic and clinical features are equally important. Dose-response studies with MTX alone in tumor-bearing mice have clearly shown that host toxicity to MTX is the major obstacle to the successful eradication of tumor by this drug. Whereas the normal stem cells of the bone marrow and gastrointestinal tract display fairly comparable degrees of sensitivity to MTX from host to host, tumor tissue displays a more variable degree of intraspecies sensitivity, and with continued treatment over a period of time the tumor usually develops drug resistance while normal tissues retain their sensitivity.

Alternate ways of coping with MTX toxicity with subsequent improvement in the therapeutic index for MTX, have included the administration of an appropriately timed antidote, such as CF, which effectively "rescues" normal cells without significantly impairing MTX's therapeutic effect (1). The clinical extrapolation of these laboratory results has been promising (2-5). However, the optimal dose and duration of both MTX and CF as well as the op-

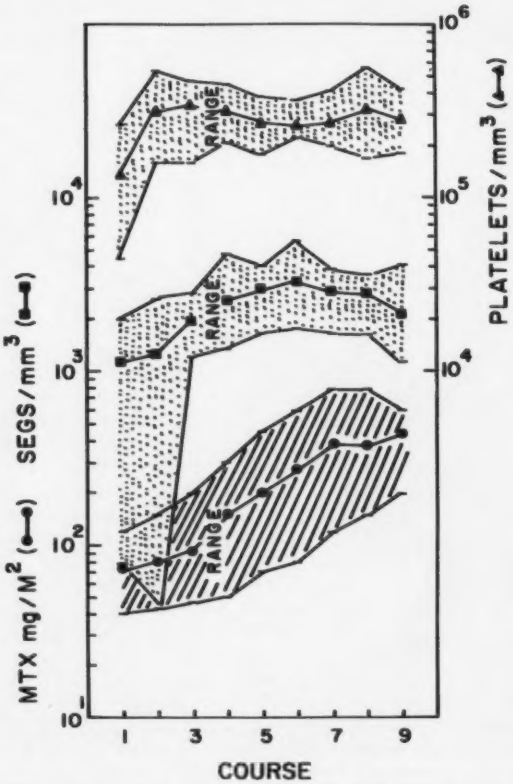

FIGURE 5.—Hematologic tolerance to MTX/L-asparaginase in 6 patients with ALL receiving 9 courses of therapy. Each point represents the mean for each group bracketed by the range of observed values. Segs = granulocytes.

timal interval between the two drugs remain to be elucidated. Another promising "rescue" possibility includes the use of a folate- and MTX-metabolizing enzyme, carboxypeptidase G_1 (12).

Preclinical observations of the effect of a protein synthesis inhibitor on the toxicity of inhibitors of DNA synthesis have made the use of MTX and L-asparaginase a logical extension from animals to man. The use of protein synthesis inhibitors has decreased the cytotoxic effect of agents like cytosine arabinoside, mechlorethamine, and X-irradiation (13).[7] The ultimate in therapeutic gain for the host would be a combination of drugs, both of which are active against the tumor and which may interact in a certain way so as to produce at the same time therapeutic synergy and toxicologic antagonism. Our laboratory and clinical results with the combination of MTX and L-asparaginase suggest that these effects may be achievable with the appropriate combination of these drugs, ie, sequence, dose, and interval. A preliminary report of the use of this combination in six adults with advanced acute lymphoblastic leukemia is very encouraging (9). These patients had not only exhausted conventional chemotherapy but had also relapsed while receiving MTX therapy. They were able to tolerate two to six times the dose of MTX when it was followed by L-asparaginase than when it was administered alone or in other combinations. In addition, all six patients entered complete remission (9). The enhanced tumoricidal effect of this combination may be the result of the host's capacity to tolerate more MTX as a result of L-asparaginase-induced toxicologic antagonism, ie, by attenuating MTX's effect on the gastrointestinal tract and bone marrow. The apparent continued therapeutic effects in leukemic mice and man may then be the result of selective rescue or cytotoxicity. Investigations are currently under way to determine which mechanism is dominant.

[7]Cytosine arabinoside: NSC-63878; CAS reg. No. 69-74-9; cytosine, 1-β-D-arabinofuranosyl-, monohydrochloride.

Mechlorethamine: NSC-762; CAS reg. No. 55-86-7; diethylamine, 2,2'-dichloro-*N*-methyl-, hydrochloride; nitrogen mustard.

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Clinical Trial of High-Dose Methotrexate (NSC-740) With Citrovorum Factor (NSC-3590)—Toxicologic and Therapeutic Observations[1,2]

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Citrovorum factor (CF) replenishes the intracellular store of reduced folates depleted by methotrexate (MTX). Thus, when used concurrently with MTX, CF will prevent the adverse biologic effects of MTX and when used after MTX CF may attenuate the biologic effects of MTX. In this setting, very large doses of MTX may be employed followed by CF rescue. The therapeutic advantage of such rescue programs was initially demonstrated in experimental systems by Goldin et al (1) and initially applied in the clinic by Djerassi et al (2). The MTX-CF rescue program is probably superior to MTX in osteogenic sarcoma, metastatic head and neck cancer, and possibly other tumors (3,4). There are several theories concerning the mechanism whereby the therapeutic index of MTX-CF may be superior to MTX alone. Certain tumor cells may be more sensitive to high concentrations of MTX, and furthermore, CF may selectively rescue normal cells as compared to certain neoplastic cells (unpublished data).

As a result of these facts and following our observations that the MTX-CF rescue program was effective in osteogenic sarcoma, we initiated a phase I and II clinical trial. A number of parallel observations were made with respect to the pharmacology of MTX (5) and to certain toxicologic manifestations, particularly those relating to, and resulting from, nephrotoxicity.

PATIENTS AND METHODS

Sixty-four patients were entered into the study (table 1): 37 adults (more than 15 years of age) and 27 children. All of these patients had biopsy-proven neoplastic disease. They had received all known effective treatment for their condition: three fourths had received prior chemotherapy and two thirds had had prior radiotherapy. All but one of the patients had had a serum creatinine of less than 1 mg/100 ml before entering the study; patients with creatinine clearances of less than 60% of the normal level (age controlled) were excluded from the study. All nonleukemic patients had had white blood cell (wbc) counts of more than 3000 cells/mm^3 and platelet counts of more than 100,000 platelets/mm^3. Sixteen of these patients had received prior MTX at conventional doses or as part of a combination chemotherapy regimen. Written informed consent was obtained from all patients.

Patients were randomly assigned to either of two MTX-CF treatment arms: a single intravenous (iv) bolus or a 20-hour infusion of MTX (fig 1). Patients receiving an infusion were given one fourth of the calculated total dose as a single push, the remainder being infused by gravity drip over the next 20 hours. CF rescue was administered 24 hours after the start of MTX administration in both groups. The first dose of 40 mg/m^2 was given iv followed by 25 mg/m^2 given orally every 6 hours for 12 doses. MTX-CF treatments were repeated at 2-3-week intervals.

Thirty-two patients received MTX by the bolus route and 32 patients received the drug by the infusion route (table 2). The initial dose of MTX was 2 g/m^2 except for "poor risk" adult patients who received 1 g/m^2. The initial dose was escalated in increments of 50% to a maximum starting dose of 6.7 g/m^2. In the absence of significant toxicity, 50% dose increments were prescribed for each patient up to a maximum of 15 g/m^2. In both infusion and bolus groups, the median number of courses for each patient was two and the median highest dose was 4.5 g/m^2.

Preservative-free MTX and CF were dissolved in sterile water for injection before iv administration. The MTX used was 83%-87% pure by weight. Water of hydration, as measured by the Carl Fischer Moisture Determination method, accounts for approximately 9% of the total weight, and of the remaining 4%-8% impurity only N^{10}-methylpteroylglutamic acid has been positively identified by paper chromatography. The latter accounts for 3% of the total impurity. The remaining contaminants have not been identified.

The group of patients whose urine was alkalinized received Scholl's Solution (100 mg Na citrate) three times/day orally and 500 mg of acetazolamide (Diamox) at bedtime.

Myelosuppression in nonleukemic patients was defined as a wbc of less than 2000 cells/mm^3 and/or a platelet count of less than 100,000 platelets/mm^3 and was considered severe if the wbc was less than 1000 cells/mm^3 and/or the platelet count was less than 50,000 platelets/mm^3.

[1]Supported in part by Public Health Service grant CA-06516 from the National Cancer Institute (NCI), National Institutes of Health, Department of Health, Education, and Welfare.

[2]Methotrexate: CAS reg. No. 59-05-2; glutamic acid, *N*-[*p*-[[(2,4-diamino-6-pteridinyl)methyl]methylamino]benzoyl]-.

Citrovorum factor: CAS reg. No. 6035-86-5; glutamic acid, *N*-[*p*-[[(2-amino-5-formyl-5,6,7,8-tetrahydro-4-hydroxy-6-pteridinyl)-methyl]amino]benzoyl]-, calcium salt; leucovorin.

All drugs used in this study were supplied by Cancer Therapy Evaluation, Division of Cancer Treatment, NCI.

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[5]The authors wish to thank Y. Bishop and C. Katsioules for their statistical support.

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TABLE 1.—Patient characteristics

Neoplastic disease	No. of patients	Age (yrs) Median	Age (yrs) Range	Sex (M/F)	Disease duration* (yrs) Median	Disease duration* (yrs) Range	No. of patients with prior— Surgery	Radiotherapy	Chemotherapy
Lymphoma	5	9	5-55	4/1	1.0	0.3-3	1	3	5
Acute lymphocytic leukemia	10	9.5	5-37	7/3	2.0	0.1-4	1	3	10
Breast cancer	6	38.5	27-70	6/0	2.5	2-12	6	6	6
Lung cancer	7	50	32-68	5/2	1.0	0.3-2	6	6	4
Head and neck cancer	8	59.5	47-78	6/2	1.3	0.1-19	3	6	3
Sarcoma	14	20	1-72	8/6	1.0	0.2-2.5	7	10	11
Acute myelocytic leukemia	3	8	3-18	2/1	0.5	0.4-0.7	0	0	3
Other†	11	29	1-57	4/7	0.8	0.1-2.3	6	8	6
Totals	64	<15 yrs (20) >15 yrs (44)		42/22			20 (31%)	42 (66%)	48 (75%)

*From diagnosis to MTX-CF therapy.
†Glioma, glioblastoma, squamous-cell vagina, squamous-cell cervix, clear-cell ovary, neuroblastoma, melanoma, thymoma, hepatoblastoma, Wilms' tumor, bladder.

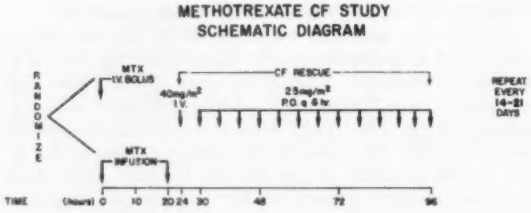

FIGURE 1.—Schematic diagram of MTX-CF study.

EVALUATION OF RENAL FUNCTION

The effect of a course of MTX-CF rescue on renal function was considered evaluable if a serum creatinine was obtained before and during the week after the administration of MTX. Since most adult patients were hospitalized for their first course, frequent serum creatinine determinations were usually made. In the second through the ninth courses, serum creatinine levels during the week after each MTX course were compared to the serum creatinine levels before the first course. The reproducibility of serum creatinine for the method in our laboratory is good: 1 standard deviation is 3.6%. For normal individuals and stable patients, multiple repetitive daily samples yielded a standard deviation of 12%. In view of this excellent reproducibility and the fact that concentrations of 10^{-3} M MTX in distilled water do not interfere with the serum creatinine determinations, a change in serum creatinine greater than 25% above the baseline was considered significant.

DEFINITION OF TUMOR RESPONSE

Complete remission was defined as a complete disappearance of all measurable disease and partial remission was defined as a greater than 50% reduction in the product of perpendicular tumor diameters. Stable disease during treatment was defined as an absence of growth of measurable disease during therapy only if preceded and/or followed by definite tumor progression.

ASSESSMENT OF MTX SOLUBILITY

MTX solubility was determined by dissolving a 1-g clinical sample (Ben Venue Laboratories, Bedford, Ohio) in a volume of distilled water just sufficient to dissolve all but a trace of solid. The concentration of MTX in the solution was calculated on the basis of quantitative ultraviolet spectrophotometric assay, using published extinction coefficient data (6). The pH of this essentially saturated solution was then adjusted from the initial value of 8.1 to 5.7 in 0.1-unit increments by the dropwise addition of 0.1 N HCl. The precipitate was centrifuged and the MTX concentration in the supernatant determined again by spectrophotometric means.

RENAL TISSUE MTX DETERMINATION

Duplicate 0.5-g samples from thawed renal cortex and medulla were prepared according to the methods of Harrap et al (7). MTX was estimated in the initial cell supernatant, before and after boiling, and in the boiled extract of the particulate material. Molarity was calculated, assuming that cell water accounts for 60% of cell volume.

TABLE 2.—MTX dose and schedule distribution

Route of administration	Age category	No. of patients	No. of courses/patient			Initial dose (g/m^2)		Highest dose (g/m^2)	
			Total	Median	Range	Median	Range	Median	Range
Bolus	Adults	23	66	2	1-9	2	1-6.75	4.5	1-15
	Children	9	23	2	1-9	4.5	2-6.75	4.5	2-9
Totals		32	89	2	1-9	3	1-6.75	4.5	1-15
Infusion	Adults	14	36	2	1-6	2	1-4.5	3	2-4.5
	Children	18	67	2	1-25	4.5	2-6.75	4.5	2-15
Totals		32	103	2	1-25	3	1-6.75	4.5	2-15

RESULTS

Nephrotoxicity

Of 37 adult patients, 33 were evaluable for renal function (table 3). Seventy-three percent of these had a serum creatinine elevation of more than 25% and 60% had an elevation of more than 50%. These 33 patients had a total of 73 evaluable courses, 47% of which were associated with an increase in serum creatinine of more than 25%.

Fourteen patients experienced an elevation of serum creatinine of more than 50% during their first course of MTX-CF (fig 2). All patients except one had a serum creatinine of less than 1.0 mg/100 ml before MTX therapy. The median baseline serum creatinine was 0.75 mg/100 ml. By Day 2, a 30% increase occurred. Peak values of serum creatinine occurred between 5 and 10 days. Three patients died with renal failure from severe myelosuppression. In the surviving patients, the median serum creatinine gradually returned toward or to normal by Day 30.

The effect of MTX dose on renal toxicity is presented in table 4. Since random allocation to initial dose was not performed and since, in general, investigators placed "poor risk" patients (elderly, with more advanced disease in terms of weight loss, tumor bulk, prior treatment, etc) on lower initial doses, the data in table 4 must be interpreted with caution. Certainly, renal toxicity is rarely seen with conventional doses of MTX and a dose response relationship between MTX and renal toxicity must be assumed. The MTX-CF rescue program produced nephrotoxic effects far more frequently in adults (58%, table 4) than in children (less than 5%, Jaffe et al [8]). The adult patients as a group had more advanced disease and host deterioration than the children; many of the adults were over age 45 and thus may have had organ function problems incidental to age. There was no correlation between increase in serum creatinine and route of MTX administration (infusion or bolus).

There was a highly significant correlation between myelosuppression and increase in serum creatinine (table 5). Thus, myelosuppression occurred in only 3% of the courses where the serum creatinine did not increase and in 55% of the courses where a 50% or greater increase in serum creatinine occurred.

Also, as seen in table 6, there was a highly significant correlation between serum MTX concentrations in excess of 1×10^{-7} M, 3+ days after MTX administration, and serum creatinine elevations of more than 50%.

The solubility of MTX in water as affected by pH is presented in table 7. Since the concentration of MTX in the urine after high-dose MTX may range up to several hundred mg/ml, precipitation might be expected to occur as the urine is concentrated and acidified in the kidneys. Two patients who died of myelosuppression associated with renal failure after the MTX-CF rescue program were observed to have bilateral renal enlargement (approximately 300 g). In one patient, the kidneys had been fixed in alcohol and an amorphous yellow material was present in the tubules. By enzymatic assay the MTX concentration was 4×10^{-4} M (0.1 mg/g) in the renal medulla and 1.8×10^{-4} M (0.05 mg/g) in the renal cortex (a concurrent serum specimen was 8×10^{-6} M). The higher concentration in the renal medulla is consistent with the fact that the medulla contains many more tubules than the cortex and supports the contention that MTX renal damage is, at least in part, the result of intratubular MTX precipitation.

In view of this, a program of alkalinization and high fluid intake (*see* Methods section) was initiated. This approach has encountered tactical difficulties related to the maintenance of alkaline urine. These preliminary studies, however, suggest that alkalinization may decrease the severity of the renal injury. While 33% of 12 nonalkalinized courses have been associated with serum creatinine elevations of more than 50%, only 18% of 27 alkalinized courses have had similar elevations.

Myelotoxicity

Eighteen (20%) of the evaluable courses of MTX-CF in adults were associated with myelosuppression as defined in table 5. As indicated in the pharmacology paper by Tattersall et al (5), there was excellent correlation between the plasma half-life and myelosuppression. Also, as seen in table 8, there was a

TABLE 3.—Frequency of renal toxicity

Increase (%) in serum creatinine	No. of patients (%) (total evaluable, 33)	No. of courses (%) (total evaluable, 73)
25-50	5 (13)	13 (18)
> 50	19 (60)	21 (29)
Totals	24 (73)*	34 (47)

* 19/24 episodes occurred with the first course.

highly significant correlation between serum MTX concentrations in excess of 1×10^{-7} M 3-4 days after the initiation of the MTX-CF rescue program, and myelosuppression. No correlation between the method of MTX administration and myelotoxicity was observed.

Gastrointestinal Toxicity

Nausea and vomiting, presumably of central origin, occurred during or immediately after MTX in 24% of the courses of MTX-CF rescue given to children. This presents a hazard if CF is to be given by mouth; in such patients, parenteral administration of CF should be continued until nausea has ceased. Nausea and vomiting rarely occurred during or immediately after MTX was given to adults.

Other Toxicity

Mucositis was rare in adult patients and occurred only in those with severe myelosuppression. As might be anticipated, there was an excellent correlation between mucositis and increased serum creatinine ($X^2 = 13.2977$, $0.001 < P > 0.01$).

Liver function abnormalities are presented in a companion paper by Jaffe et al (9).

ANTITUMOR EFFECT

One complete and four partial responses were observed in the 64 patients entered into this study (table 9). The complete response occurred in a patient with Ewing's sarcoma and has persisted for over 1 year. The ten sarcoma patients were adults with soft tissue sarcomas. If an adequate trial is defined as more than two courses of MTX-CF, there were only 20 patients who would have been considered evaluable for response.

DISCUSSION

This study of high-dose MTX with CF rescue has been discontinued in adult patients because of frequent and, at times, severe toxic reactions. The major toxic manifestations were classic for MTX and included mucositis, myelosuppression, and related complications such as infection and hemorrhage. Three patients died of nephrotoxic effects with severe granulocytopenia and sepsis. In most instances, the chain of events leading to this toxicity was initiated by depression of renal function. Nephrotoxicity, due to MTX, was described initially by Condit et al (10) but has been observed only rarely in patients receiving doses of less than 1 g/m^2. While the nephrotoxicity was manifested by more than a 25% increase in serum creatinine, in only three instances did the serum creatinine exceed 3 mg/100 ml. It should be emphasized, however, that a twofold increase in serum creatinine, for example, may represent a 70%-80% reduction in creatinine clearance. There was a highly significant correlation between the presence and magnitude of the elevated serum creatinine and the prolongation of the serum half-life of MTX; there was also a correlation between both of these phenomena and the production of myelosuppression and gastrointestinal toxicity. In all instances where it was measured, significant elevation in the serum creatinine, if it occurred, was present within 48 hours after MTX administration. Thus, both elevation of creatinine within 2 days and change in the pharmacology of MTX within the first 4 days can be used independently or together to predict the subsequent development of myelosuppression.

These observations are consistent with the fact that MTX is not metabolized and is quantitatively excreted into the urine largely by glomerular filtration (11). They are also consistent with the observation that for MTX, the duration of exposure is a substantially more important variable than the concentration in producing an adverse biologic effect. For example, the tolerated dose per unit of time in patients receiving MTX three times weekly is fivefold to tenfold greater than the tolerated dose of daily MTX (12). Thus, prolonged serum MTX levels will substantially increase toxicity.

As employed in this study, CF was reproducibly effective in rescuing and thus preventing toxicity in the bone marrow and gastrointestinal tract in those patients in whom renal damage did not occur. While we have evidence that increasing doses of CF and prolongation of CF administration may diminish toxicity in some patients with renal damage and prolonged serum concentrations, the results have been inconsistent and inconclusive. Carboxypeptidase G_1, which degrades and inactivates MTX, has proven effective in rescue programs (13).

The renal toxic effect of MTX was reversible in that the serum creatinine and, when studied, the creatinine clearance returned to normal within 20-30 days (fig 2). However, if the MTX-CF rescue program is to be used weekly, repetitive and cumulative renal toxicity would undoubtedly present a major problem.

It is evident that therapeutic trials of doses of MTX in excess of 1 g/m^2 in adults, if undertaken, will require knowledge concerning the pathogenesis and prevention of renal toxicity. To this extent maintaining an alkaline urine, particularly through the first 24-48 hours of MTX treatment, will increase

TABLE 4.—Effect of dose on renal toxicity

Dose* (g/m^2)	No. of patients	No. of patients (%) with increase in serum creatinine of—		
		25%-50%	>50%	Totals
1-2	18	3	8 (44)	11 (61)
3-6	15	2	6 (40)	8 (53)
Totals	33	5	14 (42)	19 (58)

*Represents starting dose only.

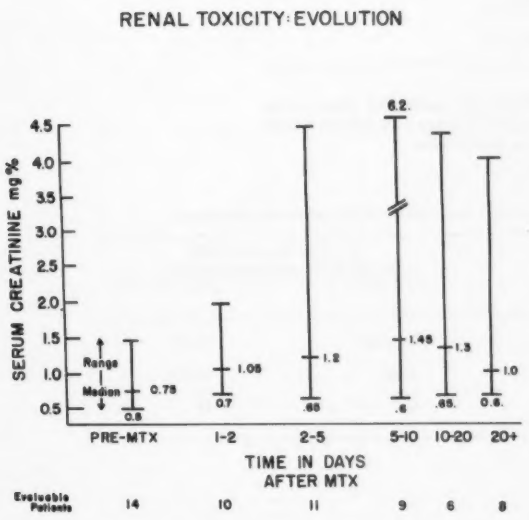

FIGURE 2.—Evolution of renal toxicity. In 14 patients there was a >50% creatinine elevation after the first course.

solubility and, to the extent that the renal lesion relates to MTX precipitation (*see* Results section), might be effective. The possibility that the folic acid antagonist effect per se is responsible for renal damage cannot be excluded. Aminopterin,[7] a folic acid antagonist closely related to MTX, is as effective in experimental systems and probably in man. However, the dose of aminopterin required is only 10% that of MTX. If the renal lesion is caused by the physical and not biochemical effects of the folic acid antagonists, aminopterin would be ideal since renal toxicity is rarely produced by MTX doses less than 0.5 g/m^2.

The therapeutic aspects of this trial are of limited significance. A significant response rate was not observed but only 20 of the 64 patients received more than two courses of treatment.

[7]Aminopterin: NSC-739; CAS reg. No. 54-62-6; glutamic acid, *N*-[p-[[(2,4-diamino-6-pteridinyl]methyl]amino)benzoyl]-.

TABLE 5.—Relation of myelosuppression to renal function in 68 evaluable courses in adults

Bone marrow function*	No. of courses	No. of courses (%) with increase in serum creatinine of—		
		<25%	25%-50%	>50%
Normal	53	35 (97)	8 (80)	10 (45)
Myelosuppressed†	15	1 (3)	2 (20)	12 (55)
Totals	68	36	10	22

*$X^2 = 21.3137$; $df = 2$; $P < 0.001$.
†Wbc count <2000 cells/mm^3 or platelet count <100,000 platelets/mm^3.

TABLE 6.—Relation of 72+ hr serum MTX molar concentration to renal function in 45 evaluable adult courses

Serum MTX concentration*	No. of courses	No. of courses (%) with increase in serum creatinine of—		
		<25%	25%-50%	>50%
$<1 \times 10^{-7}$ M	28	20 (87)	8 (100)	0
$\geq 1 \times 10^{-7}$ M	17	3 (13)	0	14 (100)
Totals	45	23	8	14

*$X^2 = 33.89$; $df = 2$; $P < 0.001$.

TABLE 7.—Solubility of MTX in water*

pH	Solubility, titrated to saturation (mg/ml)†
8.1	440 (0.97 M)
5.7	48 (0.11 M)

* pKa (MTX) = 4.8 and 5.7 (8).
† No precipitation occurred until the pH reached 5.9 when a very slight turbidity became evident; at pH 5.8, there was definite cloudiness and at pH 5.7, there was a dense precipitate.

TABLE 8.—Relation of myelosuppression to 72-96-hr serum MTX molar concentration

Bone marrow function*	No. of courses	No. of courses (%) with serum MTX concentration of—	
		$<1 \times 10^{-7}$ M	$\geq 1 \times 10^{-7}$ M
Normal	32	25 (100)	7 (53)
Myelosuppressed	8	0 (0)	8 (57)
Totals	40	25	15

*$X^2 = 16.66$; $df = 1$; $P < 0.001$.

TABLE 9.—Tumor response

Neoplastic disease	No. of patients	No. of patients evaluable*	Response†		Duration (wks)
			Complete	Partial	
Lymphoma	5	3	0	2	16+, 10+
Acute lymphocytic leukemia	10	1	0	0	
Breast cancer	6	2	0	0	
Lung cancer	7	2	0	0	
Head and neck cancer	8	3	0	0	
Ewing's sarcoma	10	3	0	0	
	4	2	1	0	56+
Acute myelocytic leukemia	3	2	0	1	8
Other	11	2	0	1‡	10

*>2 Courses MTX-CF.
†Complete response = complete disappearance of all measurable disease; partial response = > 50% reduction in tumor size.
‡ Squamous-cell carcinoma, vagina.

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Analysis of the Cytotoxic Determinants for Methotrexate (NSC-740): A Role for "Free" Intracellular Drug[1,2]

I. David Goldman[3,4]

SUMMARY

Therapeutic regimens which employ high-dose methotrexate (MTX) infusions over long intervals are likely to create conditions in which cells are exposed to levels of intracellular MTX in considerable excess of the capacity of high-affinity intracellular binding sites. This paper reviews experimental evidence which suggests that the ability of MTX to inhibit nucleic acid and protein synthesis may depend upon the accumulation of free intracellular MTX. The possibility is raised that this may be related to the association of a portion of this intracellular component with a form of dihydrofolate reductase with a reduced affinity for this agent. The therapeutic end-points and cytotoxic determinants for MTX are contrasted for conditions in which binding to the target enzyme is stoichiometric versus binding to lower-affinity target enzymes. In an accompanying paper, factors which control the intracellular MTX level in excess of the tightly bound fraction are explored, focusing in particular on the roles of membrane transport and cellular energy metabolism.

[Cancer Chemother Rep Part 3, vol 6:51-61, 1975]

The development of methotrexate (MTX) regimens which achieve high blood levels of this agent over long intervals has evolved largely from empirical observations (1-12). Although the efficacy of this approach may be accounted for within the context of the classic concept of the mechanism of action of MTX (13), recent studies employing cellular pharmacokinetic techniques (14-18) to analyze the biochemical consequences of the interaction between MTX and the intact cell suggest there may be many factors in the MTX-cell interaction, heretofore not appreciated, which may play a crucial role in the efficacy of these regimens. Broadening the understanding of the metabolic consequences of the MTX-cell interaction may help to clarify the basis for the efficacy of high-dose regimens and may most efficiently exploit the MTX-rescue approach to its full potential in the design of new treatment protocols.

CYTOTOXIC DETERMINANTS FOR MTX BASED UPON A SINGLE SITE OF ACTION: HIGH-AFFINITY DIHYDROFOLATE REDUCTASE BINDING SITES

The mechanism of action of MTX has been attributed to stoichiometric binding of this agent to high-affinity dihydrofolate reductase binding sites within the mammalian cell. Accordingly, saturation of these sites with MTX should result in the cessation of tetrahydrofolate synthesis from dihydrofolate, the depletion of cellular tetrahydrofolate-cofactor stores, and thesubsequent cessation of tetrahydrofolate-dependent biosynthetic processes, the most critical considered the synthesis of deoxythymidylate from deoxyuridylate (13). Based upon this formulation and other relevant pharmacologic considerations, the cytotoxic determinants for MTX are:

(a) The rate of MTX transport into the cell (influx).

(*b*) The capacity of the high-affinity dihydrofolate reductase binding sites within the cell. Since influx is rate limiting to intracellular binding, at any given extracellular MTX concentration the time required to saturate high-affinity sites is inversely proportional to the rate of MTX influx and directly proportional to the capacity of the high-affinity dihydrofolate reductase sites (19).

(*c*) The level of tetrahydrofolate-cofactor stores within the cell which sustain the crucial metabolic process(es) which MTX inhibits, since until these stores are depleted, the cell should survive despite the inactivation of dihydrofolate reductase.

(*d*) The activity of enzyme pathways within the cell necessary for the interconversion of tetrahydrofolate, cofactors to critical forms (ie, the conversion of 5-methyltetrahydrofolate to 5,10-methylenetetrahydrofolate).

(*e*) The presence of alternative metabolic pathways which might circumvent MTX-induced metabolic blocks (ie, the production of deoxythymidylate from thymidine [20]).

(*f*) The percentage of the cell population in S phase and the growth rate of the entire tumor cell

[1]Supported by Public Health Service grant CA-16906 from the National Cancer Institute, National Institutes of Health, Department of Health, Education, and Welfare.

[2]Methotrexate: CAS reg. No. 59-05-2; glutamic acid, *N*-[*p*-[[(2,4-diamino-6-pteridinyl)methyl]methylamino]benzoyl]-; MTX.

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population since the greater these parameters, the more potent the cytotoxic effect of MTX (21,22).

(*g*) The presence of cells in poorly perfused "pharmacologic hideouts" where the tumor cells are protected to some extent from the circulating MTX (ie, at the center of a tumor mass or shielded by the blood-brain barrier [23]).

(*h*) The rate of synthesis of "new" dihydrofolate reductase.

(*i*) The level of intracellular MTX in excess of the tightly bound fraction which is achieved within the intracellular compartment and the rate at which this intracellular component falls following cessation of MTX therapy. This consideration relates solely to the low levels of free MTX required to maintain saturation of high-affinity dihydrofolate reductase binding sites and to inhibit new dihydrofolate reductase as it is synthesized.

Within the context of these considerations, the pharmacologic endpoints for MTX are clear: (a) to achieve a sufficient extracellular MTX level for a sufficient interval to drive enough MTX into the tumor cell to saturate high-affinity dihydrofolate reductase binding sites; (*b*) to generate a *small* level of intracellular MTX in excess of these sites to saturate new enzyme as it is synthesized; (*c*) to sustain the exposure of the cells to MTX for an interval sufficient to achieve irreversible cell damage; and (*d*) to strike as large a population of cells as possible in DNA synthesis. Intrinsic to this consideration is the lack of importance of intracellular MTX in excess of the high-affinity dihydrofolate binding sites other than to meet these requirements. However, it is likely that high-dose MTX protocols result in the accumulation of appreciable levels of intracellular MTX in excess of the high-affinity dihydrofolate reductase binding sites, much more than might appear necessary to maintain saturation of these sites or inactivate newly synthesized enzyme. This paper will outline the evidence which suggests that free intracellular MTX may crucially affect the inhibition of tetrahydrofolate-dependent processes and may be one of the critical elements in the rescue protocol that accounts for its effectiveness.

ROLE OF MTX "TRANSPORT" AS A CYTOTOXIC DETERMINANT

It has been a "truism" in MTX pharmacology that the rate of translocation of MTX across the cell membrane is a crucial factor in tumor resistance. This was supported in a study by Kessel et al (24), which indicated that the percent increase in survival of mice bearing a variety of mouse leukemias was directly proportional to the uptake of MTX into the tumor cells in vitro (under conditions in which uptake represented, essentially, a unidirectional flux of drug into the cell). Indeed, while influx may very well be a crucial determinant of cytotoxicity in some tumors (25), a careful consideration of the relationship between the duration of exposure of the tumor cell to the drug (exposure time), the interval required to saturate the high-affinity dihydrofolate reductase binding sites at the extracellular MTX concentrations achieved (saturation time), and the interval over which saturation of the high-affinity dihydrofolate reductase binding sites was sustained during this interval of drug exposure (inhibition time) raises the question as to whether influx per se was the crucial determinant in this study. This is illustrated in the theoretical consideration of figure 1, which relates inhibition time as a function of MTX influx. This demonstrates that when the ratio of saturation time to exposure time is low so that inhibition time is long with respect to exposure time, the percent fall in inhibition time as influx reduces is trivial until influx falls to a very low level. For instance, when the ratio of saturation time to exposure time is 0.042, a value which is probably higher than the conditions of the study by Kessel et al in the sensitive tumors (ie, the L1210 leukemia), an 80% reduction in influx is associated with only an 18% fall in inhibition time. It is only when influx falls to much lower rates that larger decreases in inhibition times are observed, but this relationship is not linear. Indeed, proportional changes in inhibition time with a fall in influx are seen only when this ratio is more than 0.25 and approaches 0.5. Further complicating this analysis are large differences in the dihydrofolate reductase content in some of these tumors (14) which would make saturation time a variable from one tumor strain to another so that influx would not be the sole determinant of saturation time. Hence, the basis for the association of small changes in MTX influx with comparable changes in tumor susceptibility in the studies of Kessel et al is not clear. These observations raised the possibility that the apparent relationship between MTX influx and efficacy may not be attributed to changes in influx per se. Rather the crucial change might be related to another parameter of the drug-cell interaction associated with the change in influx and controlled by the same process (14).

In the pharmacology of anticancer agents, "membrane transport" is often assumed to reflect solely the rate of transport (influx) of the agent into the cell. Although the characteristics of the membrane-transport process will certainly determine the rate of MTX influx, this process will also influence the rate of exit of MTX across that membrane, the net concentration that "free" MTX achieves within the intracellular water (the concentration of that component of intracellular MTX in excess of the tightly bound fraction which is osmotically active MTX within the intracellular water), and the interval over which these concentrations are sustained. The latter parameters of the drug-cell interaction have been largely ignored because there has not been sufficient evidence for a biochemical role for this intracellular MTX component to attribute to it much pharmacologic importance. Further, in assessing the membrane transport of MTX, it must be considered that the net effect of the bidirectional fluxes of MTX (influx and efflux) will determine the free level

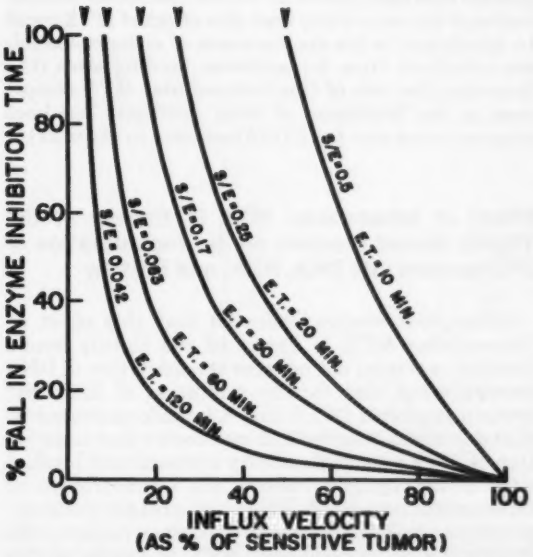

FIGURE 1.—Effect of changes in MTX influx on inhibition of high-affinity dihydrofolate reductase binding sites within the cell. This is a theoretical consideration in which it is assumed that the extracellular MTX concentration is at a level at which influx of MTX into a sensitive tumor strain saturates the high-affinity intracellular binding sites within 5 mins (saturation time). The exposure time (ET) refers to the interval over which the cells are exposed to an MTX level which achieves this saturation time, then remains sufficiently high to maintain saturation of these sites. Inhibition time refers to the interval over which the high-affinity sites are saturated with MTX. S/E refers to the ratio of saturation time to ET. The graph depicts the percent fall in inhibition time (ordinate) as influx is reduced from its initial value in a sensitive tumor strain (abscissa). This relationship is considered for five different ETs. When S/E is low, large decreases in influx only negligibly decrease inhibition time. It is only when S/E is high that a small decrease in influx is accompanied by a comparable fall in inhibition time. The arrows indicate the points at which influx is so slow that the ET is too brief to achieve total saturation of the high-affinity sites (from ref 14).

achieved within the intracellular water, and changes in one of these parameters must be accompanied by changes in one or both of the others. Hence, when influx of MTX is reduced in a cell line, this would likely be associated with a fall in the net concentration of free drug (with a concurrent fall in the interval over which levels of free drug are sustained in vivo), or a fall in efflux, or a lesser change in both parameters. Since a fall in efflux which might accompany a fall in influx would sustain intracellular MTX levels for longer intervals after cessation of drug administration and increase inhibition time, this may augment rather than decrease cytotoxicity (the role of MTX efflux is considered in the section on Roles of Drug Efflux and the Level of Free Intracellular MTX). On the other hand, a fall in the free intracellular MTX level might be associated with a decrease in cytotoxicity if there were a role for this intracellular component. The studies described in the next section were undertaken to determine whether there is a biochemical role for free intracellular MTX in the metabolic effects of this agent.

ROLE FOR FREE INTRACELLULAR MTX AS A FACTOR IN THE INHIBITION OF TETRAHYDROFOLATE SYNTHESIS AND AS A DETERMINANT OF MTX INHIBITION OF NUCLEIC ACID AND PROTEIN SYNTHESIS IN MAMMALIAN CELLS IN VITRO

Effect of Intracellular MTX in Excess of the Tightly Bound Fraction on the Incorporation of Deoxyuridine into DNA

Analysis of studies from several laboratories raised the possibility that intracellular MTX in excess of the tightly bound intracellular component may be required for maximum inhibition of DNA synthesis (14,15,26-29). We have recently evaluated this using in vitro mammalian cell systems (15-17). When cells are loaded with MTX to a level which exceeds the capacity of high-affinity binding sites, following which MTX is removed from the extracellular compartment, two components of intracellular MTX are readily discernible (fig 2). There is an initial rapid exit component and a component of intracellular MTX which remains tightly bound to high-affinity sites within the intracellular compartment. It can be shown in Ehrlich ascites tumor cells (16,30), L-cell mouse fibroblasts (15), and L1210 leukemia (31) that over brief intervals of observation (approximately 1 hour) the rate of dissociation of MTX from these high-affinity sites is negligible and may be considered essentially irreversible. Since the level of intracellular MTX in excess of the tightly bound fraction generated during the loading procedure ($\sim$2.5 μM) exceeds the K_i for the MTX high-affinity dihydrofolate reductase interaction by several orders of magnitude, it is likely that high-affinity dihydrofolate reductase binding sites are more than 99.9% saturated over this interval of observation. The following questions were then asked (15): (*a*) Was association of MTX with high-affinity sites under these conditions sufficient to inhibit DNA synthesis as measured by the incorporation of deoxyuridine into DNA? (*b*) Was there a role for intracellular MTX in excess of this component in the ability of this agent to inhibit DNA synthesis? In the experiment shown in figure 3, the effects of association of MTX with high-affinity intracellular binding sites alone on cellular DNA synthesis were assessed and contrasted with cells which contain intracellular MTX in excess of the tightly bound component. Under the conditions of this experiment, free tetrahydrofolate cofactors

were not present within the intracellular or extracellular compartments, and intracellular MTX levels were at a steady state with extracellular drug in those cells which contained free MTX in both the intracellular and extracellular compartments. It was observed that association of MTX with high-affinity intracellular binding sites alone has little effect on DNA synthesis. Indeed, the slightly reduced incorporation of tracer deoxyuridine might be related to suppression of DNA synthesis during MTX loading, with preservation of the nonlabeled intracellular deoxyuridine pool and increased dilution of the tracer added subsequently as compared to control cells which were not exposed to MTX at all. It is only when extracellular MTX is present with intracellular MTX in appreciable excess of the tightly bound fraction that there is suppression of DNA synthesis, an effect which is essentially complete at MTX levels greater than 6 μM. Figure 4 indicates that 50% suppression of DNA synthesis by intracellular MTX in excess of the tightly bound fraction requires a level of this intracellular component of approximately 0.3 μM. This effect cannot be related to penetration of intracellular compartments containing additional high-affinity dihydrofolate reductase binding sites. If that were the case, at concentrations of MTX which achieve submaximal inhibition of DNA synthesis, the rate of DNA synthesis would be expected to decrease with time as MTX entered these compartments; yet incorporation of deoxyuridine into DNA is clearly a constant function of time (fig 3). Moreover, this effect of the MTX in the second incubation cannot be attributed to inhibition of newly synthesized dihydrofolate reductase since these effects of free intracellular MTX can be demonstrated over very brief intervals (<5 minutes) (see fig 2, ref 15), and similar results are obtained when these studies are done in the presence of sufficient cycloheximide to completely abolish protein synthesis under all conditions. Other studies excluded the possibility that this effect of MTX could be attributed to the displacement of endogenous folate cofactors from intracellular binding sites (15). Recently, the role of this intracellular MTX component in the inhibition of DNA synthesis has been demonstrated also for L1210 leukemia in vitro (32).

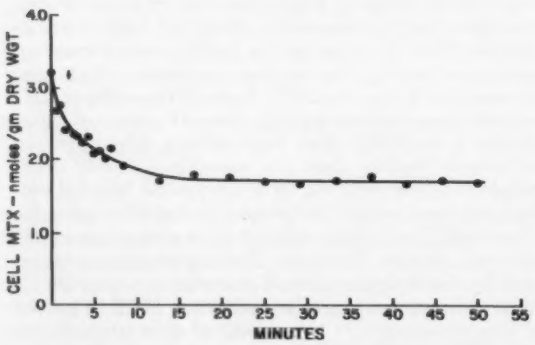

FIGURE 2.—Components of intracellular MTX. L-cell mouse fibroblasts were incubated with 6 μM MTX for 5 mins at 37°C. The cell fraction was separated by centrifugation, washed in 0°C buffer, and then resuspended into a large volume of 37°C MTX-free medium, and the fall in the intracellular MTX level was monitored. A modified Eagle's medium without serum was employed (from ref 15).

Effect of Intracellular MTX in Excess of the Tightly Bound Fraction on the Incorporation of ^{14}C-formate into DNA, RNA, and Protein

Subsequent studies indicated that this effect of intracellular MTX in excess of the tightly bound fraction is crucial not only for the inhibition of DNA synthesis but also for the inhibition of RNA and protein synthesis (17). Using a technique similar to that described in figure 3, it was shown that association of MTX with high-affinity intracellular binding sites alone negligibly effects the incorporation of ^{14}C-formate into DNA, RNA, and protein while appreciable inhibition of these processes requires the generation of intracellular MTX in excess of the tightly bound fraction (fig 5). ^{14}C-formate is used as a tracer for these biosynthetic processes since it is inserted into DNA, RNA, and protein via its association with tetrahydrofolate (33). Figure 6 illustrates the relationship between inhibition of ^{14}C-formate incorporation into RNA, DNA, and protein as a function of the intracellular MTX level in excess of the tightly bound fraction. It may be seen that 50% inhibition of formate incorporation into DNA is achieved with an intracellular MTX level in excess of the tightly bound fraction of approximately 0.3 μM, similar to the concentration that is required to produce 50% inhibition of deoxyuridine incorporation into DNA (fig 4). This suggests that inhibition of DNA synthesis as manifested by the effects of MTX on ^{3}H-deoxyuridine and ^{14}C-formate metabolism reflects an interaction between MTX and a single site affecting both processes. The concentration of intracellular MTX in excess of the tightly bound fraction required to inhibit the incorporation of ^{14}C-formate into RNA (approximately 0.8 μM) is only somewhat higher and the onset of inhibition is comparable, indicating similar sensitivities to the drug. The inhibitory effect of MTX on ^{14}C-formate incorporation into protein is much less, with 50% inhibition requiring a concentration in excess of 3 μM. The high sensitivities of DNA and RNA synthesis to MTX suggest that there are negligible tetrahydrofolate-cofactor stores to sustain these processes, while the relative insensitivity of ^{14}C-formate incorporation into protein via methionine suggests adequate cofactor stores to sustain this process. This is compatible with the observation that when cellular tetrahydrofolate production is limited, the major intracellular tetrahydrofolate cofactor is 5-methyltetrahydrofolate (34).

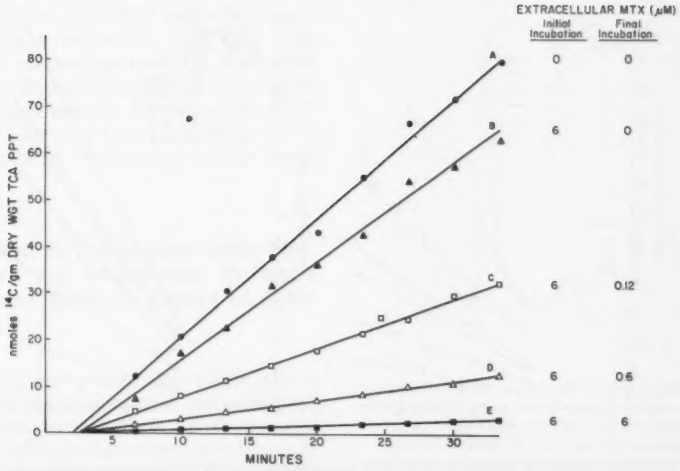

FIGURE 3.—Effect of tightly bound intracellular MTX and intracellular MTX in excess of this component on deoxyuridine incorporation into DNA. A suspension of L-cell mouse fibroblasts was divided into two portions. One portion was exposed to medium containing 6 μM MTX for 5 mins, and the other (a control) was exposed to MTX-free medium. The cell fractions were separated by centrifugation, washed with 0°C buffer, and resuspended in fresh media. The control cells (A) were again suspended in MTX-free medium, while the other cells were suspended in media in the absence of MTX (B), or in the presence of 0.12 μM MTX (C), 0.6 μM MTX (D), or 6.0 μM MTX (E). After 15 mins, the cells were separated, washed, and resuspended in fresh media of the same composition. After an additional 15 mins of incubation, when steady-state conditions for intracellular MTX had been achieved, ^{14}C-deoxyuridine was added, and the rate of incorporation of ^{14}C into the trichloroacetic acid precipitate was monitored. The final extracellular deoxyuridine level was 1.3 μM. The results were similar over a deoxyuridine concentration range of at least 0.05-1.3 μM. The medium is the same as that described in fig 2 (from ref 15).

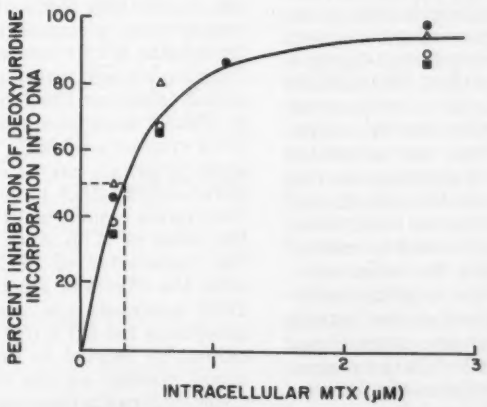

FIGURE 4.—Inhibition (%) of deoxyuridine incorporation into DNA by intracellular MTX in excess of the tightly bound fraction. This was determined from the percent depression of deoxyuridine incorporation into DNA in cells containing intracellular MTX in excess of the tightly bound fraction (lines C-E, fig 3) as compared to cells in which high-affinity binding sites were associated with MTX but intracellular MTX in excess of this level was not present (line B, fig 3). The different symbols represent four experiments performed on different days according to the methods described in fig 3 (from ref 15).

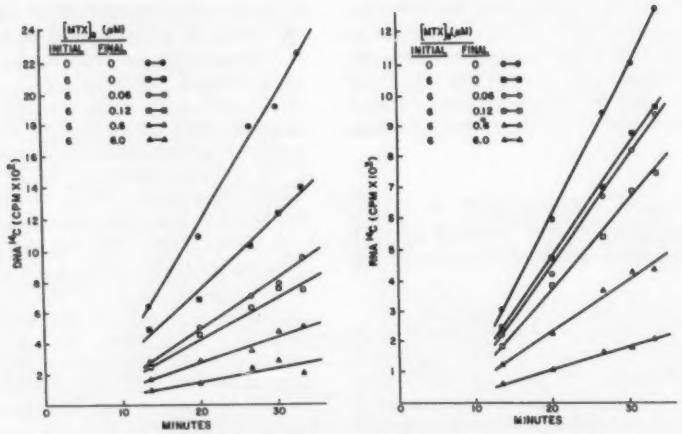

FIGURE 5.—Effect of tightly bound MTX and intracellular MTX in excess of this component on ^{14}C incorporation into RNA and DNA after exposure of cells to ^{14}C-formate. L-cell mouse fibroblasts were incubated with or without 6 μM MTX for 15 mins. The MTX-treated cells were then divided into five portions and incubated without MTX or in the presence of 0.06, 0.12, 0.6, or 6.0 μM MTX. The control cells were treated similarly, but again, were not exposed to MTX. The cells were incubated for 20 mins, washed with 0°C buffer, and resuspended into fresh media of the same composition. Ten mins later ^{14}C-formate was added to achieve a final concentration of 100 μM and cell samples were processed to determine incorporation of ^{14}C-formate into RNA (*right*) and DNA (*left*) (from ref 17).

Effect of Intracellular MTX in Excess of the Tightly Bound Fraction on the Reduction of Dihydrofolate to Tetrahydrofolate

The observation that intracellular MTX in excess of the tightly bound fraction is required for the inhibition of tetrahydrofolate-dependent processes involved in DNA, RNA, and protein synthesis suggested that this intracellular MTX component inhibits a single site which is common to the three biosynthetic processes and raised the question as to whether tetrahydrofolate synthesis, in fact, ceases when high-affinity intracellular binding sites are saturated with MTX under the conditions. Recent studies (18) suggest that there is little effect on the reduction of dihydrofolate to tetrahydrofolate under these conditions or even when cells are maintained in medium containing 0.05 μM MTX following the initial exposure to 10 μM MTX. However, when appreciable levels of intracellular MTX in excess of the tightly bound fraction are present within the intracellular component, the reduction of dihydrofolate to tetrahydrofolate can be completely abolished (fig. 7).

Stimulatory Effect of Vincristine on Intracellular MTX in Excess of the Usual Tightly Bound Fraction and the Associated Enhancement of MTX Inhibition of DNA Synthesis

The apparent critical role of intracellular MTX in excess of the tightly bound fraction in achieving the metabolic effects of this agent may account for the apparent synergism between MTX and vincristine[5] in animal systems (35), and is one element in the rationale for its use in combination with MTX in the treatment of human malignancies. Vincristine augments the cytotoxicity of MTX to the L1210 leukemia in vivo (35). Similarly, vincristine augments the steady-state accumulation of the component of intracellular MTX in excess of the usual tightly bound fraction without altering the influx of MTX in L1210 leukemia (35) or Ehrlich ascites tumor cells (30) (fig 8). This is associated with an increased inhibition of DNA synthesis by MTX while vincristine alone over short intervals has no effect on deoxyuridine incorporation into DNA in vitro (16) (fig 9). This effect of vincristine cannot be attributed to an alteration in the influx of MTX since this agent does not change this parameter of MTX transport (30). Further, since the effects of vincristine on MTX inhibition of DNA synthesis are monitored under steady-state conditions for MTX (fig 9), this effect of vincristine cannot be related to any changes that this agent might produce on the efflux of MTX. Hence, these observations further support the important role for intracellular MTX in excess of the usual tightly bound fraction in the inhibition of metabolism achieved by this agent. The stimulatory effect of vincristine on net MTX uptake might be related to the inhibition of an energy-dependent exit process

[5]Vincristine: NSC-67574; CAS reg. No. 2068-78-2; leurocristine, sulfate (1:1).

for MTX (19,36) and/or an increase in the affinity of a low-affinity receptor site for MTX capable of sustaining tetrahydrofolate reduction (16) (*see* next section). This effect of vincristine on MTX accumulation is apparently not directly related to the ability of this agent to produce mitotic arrest, since MTX uptake is unchanged over an interval of exposure to vincristine which abolishes replication of the L-cell mouse fibroblast (30).

Hypothesis: Inhibition of Tetrahydrofolate Synthesis may Require the Interaction Between Intracellular MTX and Multiple Forms of Dihydrofolate Reductase

The experimental observations described above suggest that a critical determinant of the ability of MTX to suppress tetrahydrofolate synthesis and subsequent tetrahydrofolate-dependent processes is the level of intracellular drug that is achieved in excess of the tightly bound component. The requirement for this component of intracellular MTX to achieve inhibition of these processes suggests that free intracellular MTX may be necessary to permit the interaction between this agent and a form of dihydrofolate reductase with a low affinity for MTX which sustains tetrahydrofolate synthesis when the high-affinity sites alone are saturated with MTX. The persistence of dihydrofolate reductase activity after the administration of MTX may be the basis for the observation by Mead et al that dihydrofolate reversed the lethality of MTX to mice in vitro (37).

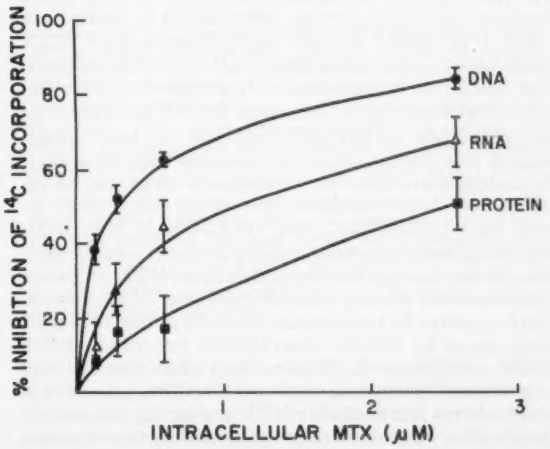

FIGURE 6.—Inhibition (%) of ^{14}C-formate incorporation into RNA, DNA, and protein by intracellular MTX in excess of the tightly bound fraction. Inhibition of ^{14}C-formate incorporation into RNA, DNA, and protein by intracellular MTX in excess of the tightly bound fraction was determined from the differences in incorporation rates between cells containing MTX equivalent to the high-affinity binding capacity and cells containing intracellular MTX in excess of this amount. This is a composite of data from experiments employing the methods described in fig 5 and ref 17.

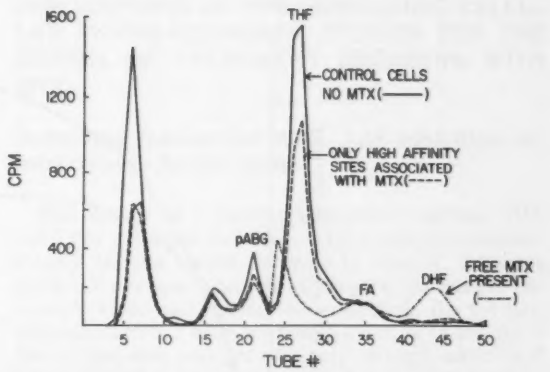

FIGURE 7.—Effect of tightly bound intracellular MTX and intracellular MTX in excess of that fraction on the reduction of dihydrofolate to tetrahydrofolate. ^{3}H-dihydrofolate was prepared by the method of Blakely (59). Ehrlich ascites tumor cells were incubated with dihydrofolate under control conditions, or with MTX equivalent to the high-affinity binding capacity or with intracellular MTX in excess of the tightly bound fraction at a steady state with 10 μM extracellular MTX, using techniques described in the legend to fig 3. After a 15-min incubation with ^{3}H-dihydrofolate in the dark at 37°C, the cells were separated by centrifugation, washed in a 0°C buffer solution, subjected to sonic oscillation, and fractionated on a DEAE cellulose column with elution by a 0-1.0 M NaCl linear gradient in tris-buffer (pH 7.2) at 4°C in the dark. Using nonlabeled markers, the following peaks were identified: dihydrofolate (DHF), tetrahydrofolate (THF), paraminobenzoylglutamic acid (pABG), and folate (FA).

Multiple forms of dihydrofolate reductase have been observed in bacteria and mammalian cells (38-46). For *Lactobacillus casei* and *Escherichia coli* at least, the affinity of dihydrofolate reductase associated with NADPH for MTX is orders of magnitude greater than the enzyme alone (44,47). Similar forms of the enzyme have been demonstrated in mammalian cells (48) and it is likely that the enzyme form unassociated with NADPH will also be shown to have a reduced affinity for MTX (48). These analyses, however, are complicated by conditions used for enzyme isolation and assay (such as a saturating level of NADPH) in cell-free systems which may result in the conversion of lower- to higher-affinity forms—a condition not relevant to the intact cell.

The effect of vincristine on MTX inhibition of DNA synthesis may be related in part to the conversion of lower-affinity to higher-affinity forms of dihydrofolate reductase. This is based upon evidence suggesting that vincristine may inhibit energy-dependent processes (30, 49-52), an effect that could increase cellular NADPH. If further studies confirm the validity of this formulation, then the level of intracellular MTX in excess of the tightly bound fraction represents at least two components, free intracellular MTX and a component which is bound to a lower-affinity dihydrofolate reductase site within the cell.

While free intracellular MTX may be necessary to interact with a low-affinity site, it is also true that

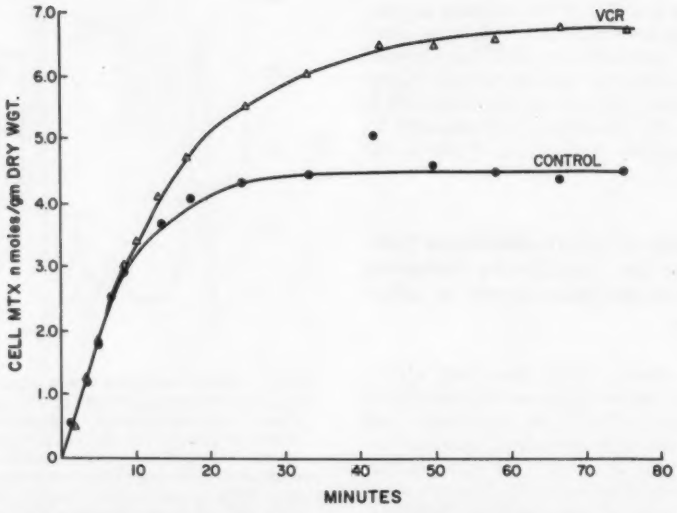

FIGURE 8.—Effect of vincristine (VCR) on the time course of MTX uptake. Ehrlich ascites tumor cells were exposed to 10 μM VCR a few seconds before addition of MTX. The extracellular MTX concentration was 0.65 μM (from ref 30).

some free MTX, albeit a small amount, will be required to maintain saturation of the high-affinity sites (*see* section on Cytotoxic Determinants for MTX Based Upon a Single Site of Action: High-Affinity Dihydrofolate Reductase Binding Sites). The recovery of deoxyuridine incorporation into DNA, as the extracellular MTX level falls, might be attributed to the dissociation of MTX from a small number of these high-affinity sites (26,28,29,53). It is uncertain what proportion of the total dihydrofolate reductase activity is required to sustain DNA synthesis. If only a small fraction of these sites supplies sufficient tetrahydrofolate to maintain this and other tetrahydrofolate-dependent biosynthetic processes, then the extent of saturation might be critical. Indeed, it is unclear as to what fraction of the total high-affinity dihydrofolate reductase binding sites are necessary to sustain near normal levels of tetrahydrofolate production. From fig 7 it can be seen that the intracellular dihydrofolate level is not detectable even when the extracellular dihydrofolate concentration is very high. Hence, the rate of dihydrofolate reduction to tetrahydrofolate must be quite rapid in comparison to the rate of transport into the cell. If the intracellular dihydrofolate level is in the range of or below its K_m for dihydrofolate reductase in these experiments, which seems quite likely, association of MTX with a large percentage of high-affinity dihydrofolate reductase sites would be expected to have only small effects on tetrahydrofolate production (Jackson and Harrap (54) estimated that only 5% of dihydrofolate reductase activity would be required to sustain near normal rates of tetrahydrofolate synthesis in the L1210 leukemia). However, the experimental design employed in these studies achieves initial intracellular MTX levels 3-4 orders of magnitude above the MTX -dihydrofolate reductase K_i (17) and tight binding appears essentially irreversible under these conditions (see fig 2). Further, reversal of inhibition of deoxyuridine incorporation into DNA occurs within 5 minutes after cells are resuspended into MTX-free medium (fig 2, ref 15). Hence, it seems unlikely that the very small number of high-affinity dihydrofolate reductase sites that might remain unassociated with MTX even under these conditions ($< 0.1\%$) could account for sustained tetrahydrofolate synthesis. However, since the dissociation constant for MTX-dihydrofolate reductase *within* these cells has not been established, and the relationship between enzyme activity, tetrahydrofolate production and the rates of tetrahydrofolate-dependent reactions have not, as yet, been quantitated, final resolution of this problem must await further study. Irrespective, however, of the mechanism by which free MTX achieves the observed effects, this intracellular MTX component appears to be a crucial element in the ability of this agent to inhibit biosynthetic processes under some conditions. It follows then that the cellular regulatory processes and external factors which control free intracellular MTX and/or the characteristics of a "lower-affinity" dihydrofolate reductase site may be important determinants of the cytotoxicity of MTX. This is considered further in detail in the accompanying paper (55).

A recent study suggested that saturation of high-affinity intracellular binding sites with MTX in L1210 leukemia cells in vivo may be sufficient to achieve inhibition of DNA synthesis (32) under the reported experimental conditions. The possibility

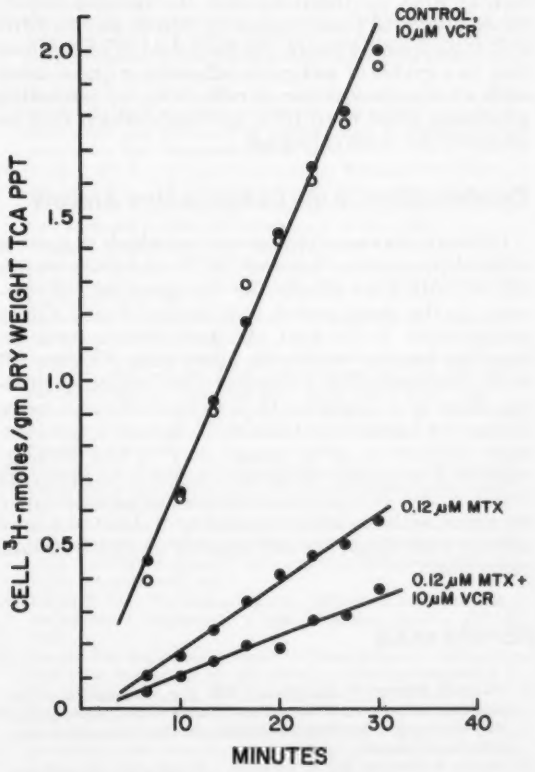

FIGURE 9.—Effect of vincristine (VCR) on MTX inhibition of deoxyuridine incorporation into DNA. Cells were divided into two portions, one a control suspended in MTX-free medium and the other exposed to 6 μM MTX. After a 5-min incubation at 37°C, the cells were separated by centrifugation and washed twice with 0°C buffer. Control cells were resuspended into MTX-free medium, and the cells exposed to MTX were resuspended into medium containing 0.12 μM MTX. The two suspensions were then further divided in half, one portion of which was exposed to 10 μM VCR. After a 15-min incubation at 37°C, all cell suspensions were exposed to ^{3}H-deoxyuridine at 0 time and the rate of incorporation of ^{3}H into DNA was monitored. The upper line indicates control cells and cells exposed to 10 μM VCR without MTX. The middle line reflects those cells incubated with 0.12 μM MTX. The lower line indicates the cells exposed to 0.12 μM MTX plus VCR. The medium was as described in fig 2 (from ref 15).

that cytotoxic determinants for intracellular MTX in vitro may differ from those in vivo will certainly require further examination. Indeed, the presence or absence of low-affinity dihydrofolate reductase forms as an alternative source of tetrahydrofolate synthesis or differences in the binding constant of a single dihydrofolate reductase specie in the in vitro or in vivo condition may be crucial factors in MTX cytotoxicity. This may account for the efficacy of some low-dose MTX regimens which might not be expected to achieve appreciable levels of free intracellular MTX.

IMPLICATIONS OF THE FOREGOING CELLULAR PHARMACOKINETIC STUDIES FOR THE DESIGN OF TREATMENT REGIMENS WITH MTX

Achieving Interaction With, and Inhibition of, Intracellular Target Site(s)

The design of a chemotherapeutic regimen with MTX for a system in which MTX binds stoichiometrically to the target enzyme is clearly different from the system in which binding to the target enzyme is loose and rapidly reversible. In the former, the endpoint for drug administration is to saturate the target site and provide only enough additional intracellular drug in excess of this level to sustain saturation of the site and inhibit newly synthesized enzyme. This is an endpoint that can be achieved by generating only small amounts of intracellular MTX in excess of the tightly bound fraction, and should be sustained for long intervals following cessation of drug therapy. On the other hand, the endpoint to achieve inhibition of a lower affinity site would require (a) pumping substantially greater amounts of MTX into the cell in excess of that required to saturate the high-affinity sites, and (*b*) sustaining this level for the critical interval necessary to produce sufficient damage to the cell such that reversal will not occur after cessation of the MTX infusion and the administration of 5-formyltetrahydrofolate during the "rescue." Obviously, the delivery of the drug to *all* the tumor cells and its ability to penetrate membrane barriers remain essential factors, since generation of free drug cannot be accomplished until these endpoints are satisfied.

Roles of Drug Efflux and the Level of Free Intracellular MTX

Beyond achieving inhibition of high- and low-affinity intracellular sites, the absolute level of free intracellular MTX achieved during the MTX infusion will influence the level of free drug within the cell as the extracellular MTX level falls with time after the MTX infusion is stopped. If the critical interval which produces lethal cell damage is not achieved during the administration of MTX, then the fall in the level of free drug following cessation of therapy may be an important determinant of cytotoxicity. Under these conditions, when the net rate of drug exit is slow and/or the initial drug concentration is high, a sufficient drug level may remain within the cell for a sufficient interval to achieve the lethal effect. However, it may be inefficient and dangerous to strive for very high intracellular MTX concentrations over long intervals merely to extend the time over which there are critical levels of free drug within the cell after the drug is discontinued. Rather, a logical approach would be (a) the administration of an initial high dose of MTX to achieve a

blood level sufficient to "drive" MTX into poorly perfused tumor masses and low-permeability membrane barriers to generate a "critical" free intracellular MTX concentration, followed by (*b*) a reduction in the MTX blood level to a concentration that will *maintain this critical intracellular MTX concentration* for a sufficient interval of drug infusion. In many high-dose MTX regimens it is unlikely that free intracellular MTX reaches thermodynamic equilibrium with extracellular MTX over short intervals. Rather, even when the intracellular MTX level is sufficient to abolish tetrahydrofolate synthesis, it is probable that there is a very high gradient for extracellular/intracellular free MTX, and the maintenance of this "critical " free intracellular MTX concentration could be accomplished with much lower extracellular MTX concentrations. Hence, once the initial high load of MTX has penetrated membrane and diffusional barriers, the drug may not diffuse or be transported out of the cell even when the extracellular drug level is reduced to levels much lower than that required to initially generate the "critical" free intracellular MTX level.

Implications of the Relative Inhibitory Effects of MTX on Tetrahydrofolate-Dependent Processes with Respect to MTX Cytotoxicity

The cytotoxic effects of MTX are usually attributed to the ability of this agent to inhibit DNA synthesis. Yet there are conflicting data as to whether the consequences of inhibition of DNA synthesis by MTX are influenced by the degree to which there is concurrent inhibition of RNA synthesis. In one report, when RNA synthesis continued after DNA synthesis was blocked and there was "unbalanced" nucleic acid synthesis, MTX cytotoxicity was augmented (56), while other reports suggested that the purineless state induced by MTX may be a crucial determinant of cytotoxicity (57,58). When the effects of MTX on tetrahydrofolate-dependent processes were quantitatively compared in the studies described in the section on Effect of Intracellular MTX in Excess of the Tightly Bound Fraction on the Incorporation of ^{14}C-formate into DNA, RNA, and Protein (fig 6) (17), differences in the levels of MTX required to inhibit these processes were observed. DNA synthesis was most sensitive to MTX, the sensitivity of RNA synthesis was somewhat less though in a comparable range. Incorporation of ^{14}C-formate into protein was much less sensitive to MTX. It must be considered in the design and evaluation of high-dose MTX protocols that the degree to which these processes will be concurrently inhibited is uncertain; one, two, or all might be inhibited. Hence, depending on the intracellular drug levels achieved and the characteristics of the particular tumor, there may or may not be unbalanced inhibition and this may be an important factor in determining the efficacy of the protocol. Further, while the toxic effects of MTX are usually attributed to inhibition of DNA synthesis (13), it is likely that there is often concurrent inhibition of RNA synthesis so that the relative importance of each of these inhibitory effects may be difficult to decipher. Finally, the high-dose MTX regimen may be capable of adversely affecting a tumor mass with a large proportion of cells in G_1 by inhibiting processes other than DNA synthesis which may be lethal to the nondividing cell.

Considerations in the Design of New Antifols

Cellular pharmacokinetic studies which suggest a critical interaction between MTX and sites within the cell with a low affinity for this agent may be relevant to the development and design of new folate antagonists. In the past, the goal of structural design has been to develop an agent with a higher affinity for high-affinity dihydrofolate reductase binding sites or a capacity to penetrate the cell membrane at a higher rate than MTX. However, an alternate endpoint to drug design may be the development of a compound which accumulates to high free levels within cells (*see* accompanying paper [55]) or an agent with an enhanced ability to bind to a low-affinity site within the cell capable of dihydrofolate reduction.

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Membrane Transport of Methotrexate (NSC-740) and Other Folate Compounds: Relevance to Rescue Protocols[1,2]

I. David Goldman[3,4]

SUMMARY

This paper (a) reviews the properties of the membrane transport of methotrexate (MTX) and the tetrahydrofolate cofactors, (b) considers the factors that control the level of free intracellular MTX, and (c) analyzes the possible consequences of the interaction between these substances at the level of the cell membrane that may influence the effectiveness of "rescue" protocols. This approach may facilitate the design of more effective treatment regimens employing high doses of MTX with folinic acid "rescue."

[Cancer Chemother Rep Part 3, vol 6:62-72, 1975]

The mechanism(s) of the membrane transport of methotrexate (MTX), folic acid, and the tetrahydrofolate (THF) cofactors (5-methylTHF and 5-formylTHF), and the relationship between translocation of these substances across cell membranes and their subsequent intracellular disposition in mammalian cells, represent complex processes that have not, as yet, been completely defined and continue to be studied (1-19). While there has been considerable interest in the transport of these compounds when their extracellular concentrations are low, conditions relevant to the physiologic transport mechanism and the usual MTX levels achieved with low-dose regimens, there is at present little information regarding the characteristics of transport at levels achieved in high-dose MTX protocols. This paper will *(a)* review the properties of the membrane transport of MTX and the THF cofactors as they are currently understood, and *(b)* present a quantitative conceptualization of the possible effects of MTX-THF cofactor interactions at the level of their common membrane carrier that may influence the effectiveness of rescue protocols. This may provide insight into critical factors in treatment protocols that are not intuitive and may permit increasingly rational designs for new regimens.

HIGH-AFFINITY TRANSPORT CARRIER FOR MTX AND THF COFACTORS

A high-affinity transport system for MTX has been established in a number of mammalian cells which conforms to a carrier model that MTX shares with 5-methylTHF and 5-formylTHF (12,16). Influx of MTX is saturable with a K_t (MTX concentration at which influx is 50% of maximum) in the range of 5 μM and a maximum influx velocity in the range of 5 nmols/g dry weight of cells/minute (fig 1). Similarly, the influxes of 5-methylTHF and 5-formylTHF are saturable, with K_t's in the same range as MTX (12,14). Each compound inhibits the influx of the other and participates in heteroexchange interactions (16): *(a)* The presence of 5-formylTHF or 5-methylTHF within the intracellular compartment accelerates the unidirectional influx of MTX into the cell (a "transconcentration effect"). *(b)* The addition of either of these THF cofactors to the extracellular compartment when the cells are at the steady state with MTX leads to a net flow of free MTX out of the cell. Likewise, the addition of MTX to the extracellular compartment when the cells are at a steady state with 5-formylTHF or 5-methylTHF leads to a net flow of these THF cofactors out of the cell (14) (a "countertransport" phenomenon) (16). These observations not only confirm that these compounds share a transport carrier but illustrate ways in which they may affect the transport of each other during rescue protocols—competitive interactions at the cell membrane which may influence efficacy of the treatment regimen. In addition to these transport characteristics, MTX influx is *(a)* highly temperature sensitive, *(b)* pH sensitive, and *(c)* markedly inhibited by sulfhydryl reagents (7,8,18).

The energetics of MTX accumulation within the cell conform in some ways to other active transport processes but in other respects are unusual. At low MTX concentrations, the level of intracellular MTX in excess of the tightly bound fraction (to be referred to as "exchangeable intracellular MTX") exceeds the extracellular MTX concentration (8). However, chemical gradients alone are insufficient to assess the capacity of cells to generate uphill transport of MTX because this agent is anionic at phys-

[1]Supported by Public Health Service grant CA-16906 from the National Cancer Institute, National Institutes of Health, Department of Health, Education, and Welfare.

[2]Methotrexate: CAS reg. No. 59-05-2; glutamic acid, *N*-[*p*-[[(2,4-diamino-6-pteridinyl)methyl]methylamino]benzoyl]-; MTX.

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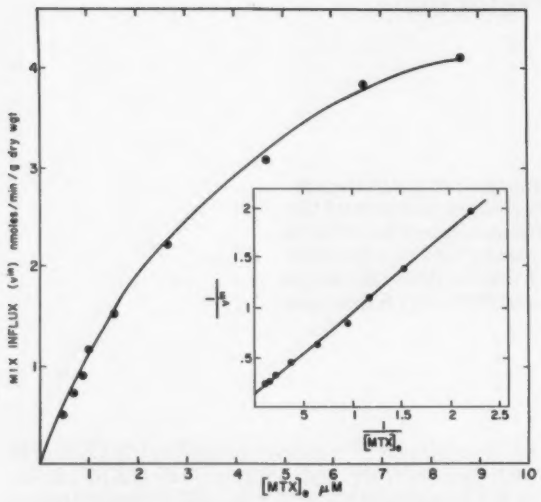

FIGURE 1.—Relationship between MTX influx and extracellular MTX concentration ($[MTX]_e$) in L1210 murine leukemia cells. MTX influx is determined by measuring the net uptake of ^{3}H-MTX into cells over an interval during which the high-affinity binding sites remain unsaturated. [Reprinted with permission from ref 8.]

iologic pH and the cells carry a negative charge. Unfortunately, the membrane potential has not been established in most tumor cells studied so that the actual electrochemical-potential difference for MTX across the cell membrane is uncertain. However, it would appear that some cell lines sustain a higher intracellular than extracellular electrochemical-potential for MTX, suggesting an energy-dependent uphill process (8). Analysis of the energetics of this transport system is further complicated by the effects of metabolic inhibitors which enhance rather than reduce net accumulation of MTX (2,10,12,15). This appears to be related, at least in part, to an increase in the electrochemical-potential for free intracellular MTX but may be due, in addition, to an alteration in MTX binding to intracellular constituents (20). This is discussed in more detail in the accompanying manuscript (21). Apparent augmentation of uphill MTX transport into the cell by metabolic poisons would be attributed to the inhibitory effect of these agents on an exit pump for MTX (10,-12).

Another characteristic of MTX transport as it was studied at low concentrations was the observation that a variety of inorganic and organic anions when added to the extracellular compartment inhibit the influx and steady-state intracellular electrochemical-potential for MTX (12). It was proposed that the usual gradient for organic phosphates across mammalian cell membranes which results from their synthesis within the intracellular compartment and their relative inability to penetrate cell membranes might be the basis for a counter-transport phenomenon in which the downhill flow of organic phosphates out of the cell via the very low-capacity MTX carrier results in the uphill flow of MTX into the cell. Since the ability of the organic phosphates to interact with the MTX carrier is independent of their energy potential, this may be the mechanism by which uphill transport of MTX into the cell is sustained over short intervals after exposure to metabolic poisons and cessation of the synthesis of high-energy intermediates (12).

CHARACTERISTICS OF MTX UPTAKE AT HIGH EXTRACELLULAR MTX CONCENTRATIONS

In the past, studies on the membrane transport of MTX have utilized low drug concentrations which reflect the characteristics of the high-affinity carrier-mediated route. This may be attributed to the following considerations: *(a)* At the doses of MTX employed in most chemotherapeutic regimens, the extracellular MTX concentrations achieved are in the range of the K_t for the high-affinity carrier route so that transport of MTX would be mediated almost exclusively by this system and the properties of this route would be most relevant for study. *(b)* MTX has been utilized as a "model" substrate (8,12) to explore the transport properties of the naturally occurring THF cofactors (especially 5-methylTHF, the major extracellular folate of man and rodents [22,23]), based upon the ease of purification and the stability of tritiated MTX, its availability at high specific activity, and the lack of metabolism in many cells over short intervals of exposure (8,11,13,15,24). Since MTX and 5-methylTHF have comparable affinities for the high-affinity carrier, and usual blood levels for 5-methylTHF are below the influx K_t for this compound, the properties of transport relevant to the way 5-methylTHF traverses cell membranes under physiologic conditions have required evaluation at low MTX and 5-methyl THF levels. The relevance of these observations, however, to the properties of MTX transport at concentrations orders of magnitude above this carrier K_t is uncertain. Hence, with the advent of high-dose MTX protocols and the high extracellular levels of drug achieved, the properties of MTX transport under these conditions assume greater importance and will demand further study.

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[5]Goldman ID, and White JC. Unpublished observations.

While cellular uptake of MTX continues even after the high-affinity carrier is saturated,[5] there is little information on the characteristics or significance of this cellular uptake component. It would be expected that as the MTX level is increased, passive diffusion of drug would become a more important component of the total flux across the cell membrane, but this has not been quantitated. Further, a low-affinity transport carrier could contribute to MTX uptake at high drug concentrations. Clearly, the properties of MTX transport at high concentrations will deviate from those described for the high-affinity carrier to the extent that transport under these conditions is dominated by passive diffusion and/or a low-affinity carrier. The interaction between MTX and other folates and the energetics of transport under these conditions will require further study.

RELATIONSHIP BETWEEN THE MEMBRANE TRANSPORT OF MTX AND OTHER FOLATE COMPOUNDS

It is clear that in several mammalian cells studied, MTX, 5-methylTHF, and 5-formylTHF share in common a high-affinity transport carrier (12). This is supported by competitive and heteroexchange interactions (see previous section). In addition, as observed for MTX, the influx of 5-methylTHF is inhibited by anionic compounds (25). The data for folic acid are more difficult to interpret so that the relationship between the transport of this compound, MTX, and the THF cofactors is unclear (8,-9,11,12,14,18,19). This is an issue which is of more academic than practical interest since the folic acid blood level is so low and folic acid transport into cells so slow that its rate of delivery into peripheral tissues could not be important in providing folate substrate for THF-dependent processes in comparison to the rate of delivery of THF cofactors (9,12). Be that as it may, folic acid is a weak inhibitor of MTX influx into Ehrlich ascites and L1210 leukemia cells at MTX concentrations below the high-affinity carrier K_t for the drug and folic acid participates in hetero-exchange phenomena with MTX (8,-9,11,12,14,19). On the other hand, inhibition of the initial uptake velocity for equimolar folic acid is less than expected when the MTX concentration is above the K_t (10 μM) when there should be a high degree of carrier saturation by MTX.[6] This suggests that while folic acid has a very low affinity for the "high-affinity" MTX-THF carrier, it can inhibit MTX transport by this route when present at sufficiently high concentrations. However, although folic acid may also utilize this route to a small extent when present at these extracellular concentrations ($\sim$10 μM), this substance largely traverses the cell membrane by another process. This is also suggested by the observation that sulfhydryl inhibitors abolish MTX influx but have little effect on folic acid uptake (18).

REGULATION OF THE EXCHANGEABLE INTRACELLULAR MTX LEVEL

The accompanying paper (21) describes evidence which suggests that not only is a sufficient rate of MTX influx into the cell and the consequent rate of inactivation of high-affinity dihydrofolate reductase binding sites prerequisites for MTX cytotoxicity but, in addition, the exchangeable intracellular MTX level may be a critical cytotoxic determinant. This is based upon data which suggest either *(a)* an interaction between a component of intracellular MTX in excess of the tightly bound fraction and a form of dihydrofolate reductase with a low affinity for MTX and/or *(b)* nonstoichiometric binding of MTX to the "high-affinity" site. In either case inhibition of tetrahydrofolate production would require appreciable concentrations of free intracellular MTX, the level of which will be determined by the characteristics of the membrane transport mechanism(s), cell energetics, and other physical properties of the cell. This will be considered in the following section.

Relationship Between the Extracellular MTX Concentration and the Exchangeable Intracellular MTX Level

When a carrier-mediated transport process is coupled to cellular energy metabolism, not only is the influx of the transported substance saturable but, in addition, the concentration that the substance achieves within the intracellular water may be a saturable function of the extracellular concentration (as in an adsorption isotherm) (16). This occurs when energy coupling results in saturation of efflux at a higher concentration than influx. In the case of MTX transport, when the extracellular MTX level is in the range of the high-affinity carrier K_t, the exchangeable intracellular MTX level is a saturable function of the extracellular drug concentration, an observation that may be attributed to the kinetics of transport (fig 2). Although this could also be related in part to the binding of MTX to low-affinity sites within the cell, it is unlikely that loose binding is the major factor in these uptake kinetics since exchangeable intracellular MTX does not markedly increase above predicted values, based upon the high-affinity carrier, even at very high extracellular MTX levels. Thus, the characteristics of the high-affinity transport route may limit the maximum level of free intracellular MTX and in this way may be important determinants of cytotoxicity.

If there is one carrier route for a substance across a membrane, then there must be at least one other parallel transport process, passive diffusion, but the magnitude of this component will depend entirely upon the concentration, size, lipid solubility, and

[6] See footnote 5.

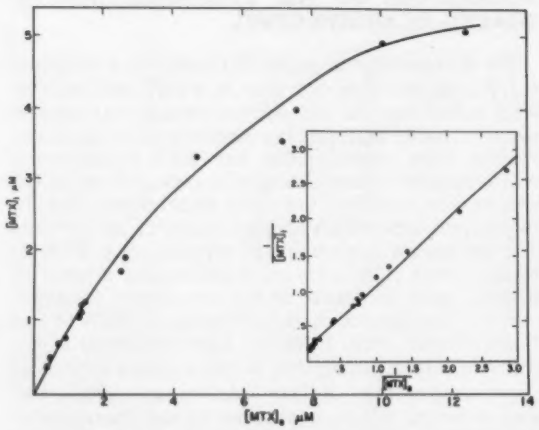

FIGURE 2.—Relationship between the steady-state exchangeable intracellular MTX level ($[MTX]_i$, and the extracellular MTX concentration ($[MTX]_e$. Exchangeable intracellular MTX is determined from the difference between the total-cell MTX level and the component of MTX which is tightly bound as determined from the level of MTX which is tightly bound as determined from the level of MTX which remains in cells after they are suspended into a large volume of MTX-free medium. [Reprinted with permission from ref 8.]

the charge of the substance, as well as the properties of the cell membrane. If the carrier system is coupled to energy metabolism and sustains a gradient for the substance across the cell membrane, then the passive transport component will act as a "leak" that will dissipate the electrochemical-potential difference created by the carrier. Hence, when the concentration of the substance is very high, and the "leak" is appreciable, the electrochemical-potential difference for that substance across the cell membrane will be eliminated (16). For MTX, as the extracellular level is increased and the carrier system saturates, the ratio of the exchangeable intracellular to extracellular MTX concentration falls to very low levels as the intracellular MTX level approaches its maximum (8). The capacity of cells to achieve higher intracellular MTX concentrations will depend upon the magnitude of the passive diffusional transport component and/or the presence of a low-affinity transport route. In high-dose MTX regimens which generate MTX blood levels orders of magnitude above the high-affinity carrier K_t and completely saturate that route, transport by passive diffusion or by a low-affinity carrier may be the critical determinant of both the rate of MTX influx and the steady-state level of MTX achieved within the cell. However, if over a broad concentration range, the high-affinity carrier is the only important transport route for MTX, then exceeding an extracellular drug level which achieves more than 90% of the maximum intracellular level would be of little value and might only serve to drive MTX into normal host tissues with different transport properties.

Role of Cellular Energy Metabolism in the Control of Exchangeable Intracellular MTX: Effect of Vinca Alkaloids

When the level of exchangeable intracellular MTX is limited by the properties of the high-affinity carrier and when alternate transport pathways are either negligible or cannot practicably be utilized, then alterations in the exchangeable intracellular MTX level can be achieved by either changing the membrane potential (see below) or by altering cellular energy metabolism. Virtually any metabolic poison augments the level of exchangeable intracellular MTX (10); the ability of vinca alkaloids to induce the same changes in exchangeable MTX may be related to the capacity of these agents to alter energy-related processes (15,26). The effect of vincristine[7] in particular has been studied in detail (15). While this agent does not alter the influx of MTX (15), vincristine augments MTX inhibition of DNA synthesis (20) and increases MTX cytotoxicity to the L1210 leukemia in vivo (27), supporting the critical role of the exchangeable intracellular MTX level in achieving the metabolic consequences of this agent (this is discussed in detail in the accompanying paper [21]). However, the ability of vincristine to augment the exchangeable intracellular MTX level may be useful only under certain conditions, ie, when the exchangeable intracellular MTX level achieved in the tumor cell is inadequate to suppress THF-dependent reactions and the magnitude of the increase in this component induced by vincristine is sufficient to depress these processes. On the other hand, if tumor cell resistance is due to a markedly low rate of MTX influx so that high-affinity dihydrofolate reductase binding sites are not saturated and exchangeable intracellular MTX does not accumulate at all, then vincristine might not be expected to have any effect on cytotoxicity since this agent does not alter MTX influx (see fig 3 and ref 20). The potential usefulness of vincristine with very high-dose MTX regimens, when alternate transport pathways might be utilized, will require study as will the relative effects of this agent on tumor versus normal susceptible host tissues. In experimental systems, at least, there is variability in the response of different cell lines to vincristine (15), a factor that could be of value in optimizing the therapeutic index for this drug concentration.

Since the usefulness of vincristine in this context depends upon many elements of the vincristine-MTX interaction with host and tumor cells, the observation that vincristine does not potentiate the effect of MTX in some regimens or in some patients does not exclude a role for this combination in all regimens or in all patients. Clearly, cellular pharmacokinetic (21) analyses for specific kinds of tumors and tumors

[7]Vincristine: NSC-67574; CAS reg. No. 2068-78-2; leurocristine, sulfate (1:1).

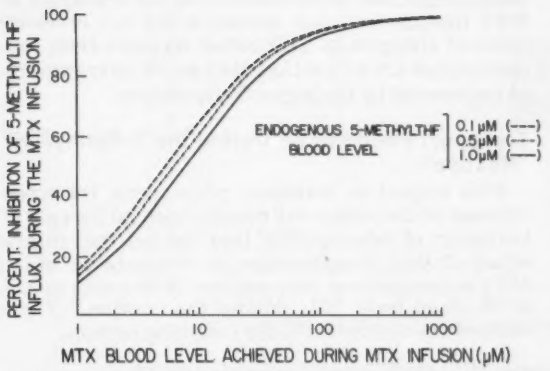

FIGURE 3.—Effect of MTX on endogenous 5-methylTHF influx into cells during the MTX infusion. The graph represents a computer analysis for MTX levels between 1 μM and 1mM at three different 5-methylTHF levels, 0.1, 0.5, and 1.0 μM. Kinetic constants utilized in this analysis were taken from experimental cell systems (12-14). Percent inhibition was computed from:

$$\left(\frac{\left(1 + \frac{K_t}{[5\text{-methylTHF}]_e}\right)\left(1 + \frac{[MTX]_e}{K_i}\right)}{\frac{[5\text{-methylTHF}]_e}{K_t + [5\text{-methylTHF}]_e}}\right)(100)$$

where $[5\text{-methylTHF}]_e$ is the extracellular 5-methylTHF concentration, K_t is the level of $[5\text{-methylTHF}]_e$ required to half-saturate the influx mechanism (estimated at 2 μM), and K_i is the transport inhibitor constant for MTX (estimated at 5 μM).

from specific patients may be necessary before the full potential of combined vincristine-MTX regimens can be realized.

Effect of Membrane Potential on the Free Intracellular MTX Level

Since MTX is anionic at physiologic pH, the concentration that MTX achieves within the intracellular water will depend upon the membrane potential (see section on High-Affinity Transport Carrier for MTX and THF Cofactors). Given either *(a)* a non-energy-dependent transport mechanism or *(b)* a coupling of energy to a transport carrier resulting in an electrochemical-potential difference for MTX across the cell membrane which is independent of the membrane potential, the higher the membrane potential (ie, the more negative the cell), the lower the chemical concentration of MTX within the intracellular water. Conversely, the lower the membrane potential (ie, the less negative the cell), the higher the intracellular MTX concentration. Hence, conditions or agents which alter the membrane potential may alter the concentration of free MTX, or any other charged folate, within the intracellular compartment. There is little information, however, on conditions causing membrane potential alterations that might be exploited clinically to enhance intracellular MTX accumulation by this mechanism. Similarly, structural modifications of the MTX moiety which eliminate its negativity, such as diesterification (28), or produce a positive charge would increase the steady-state free intracellular drug level and in the latter case produce a chemical gradient (intracellular/extracellular) of free drug across the cell membrane even under conditions in which transport is passive.

Effect of the Anionic Composition of the Extracellular Compartment on the Free Intracellular MTX Level

The influx of MTX and the steady-state intracellular electrochemical-potential for this agent are reduced by addition of organic and inorganic anions to the extracellular compartment (12). MTX transport can be influenced by *(a)* virtually any anionic drug if it is present in sufficient concentration, or *(b)* retention of endogenous inorganic or organic anions as in uremia (25). By altering the membrane transport of MTX, these conditions may alter cytotoxicity, but it is unclear at present as to whether this is of clinical importance. Further, in assessing the potential influence of these anionic interactions on MTX cytotoxicity, other factors should be considered such as *(a)* displacement of MTX from plasma protein binding sites, or *(b)* competition for the renal secretory mechanism (29). The latter factors would increase the free MTX blood level increasing MTX transport into the cell, thus tending to diminish or reverse the anionic inhibition of transport at the level of the cell membrane.

Interactions Between MTX and THF Cofactors at the Level of the Cell Membrane in "Rescue" Protocols

During the high-dose MTX infusion and subsequent 5-formylTHF "rescue," interactions between MTX, 5-methylTHF, and 5-formylTHF, as well as other THF cofactors at the level of their common membrane carrier(s), may influence the effectiveness of the regimen. In this section, these factors are analyzed based upon data obtained from studies in experimental mammalian cell systems (1-19).

Transport Phenomena During the MTX Infusion

The extent to which MTX inhibits THF-dependent processes during the MTX infusion will depend upon *(a)* transport of sufficient MTX into the cell to inactivate its target site(s), and *(b)* inhibition of 5-methylTHF transport from the extracellular compartment into the tumor cell so that there is an inadequate delivery of this cofactor to sustain folate-dependent reactions after THF synthesis ceases and

cellular cofactor stores are depleted. When the levels of MTX introduced into the extracellular compartment are high enough to saturate the high-affinity carrier, influx of 5-methylTHF will be reduced since endogenous 5-methylTHF blood levels are below the carrier K_t for this THF cofactor. The relationship between the extracellular MTX level achieved during the infusion and the expected inhibition of 5-methylTHF influx into the cell is illustrated in figure 3 for three different 5-methylTHF blood levels. It may be seen that 50% inhibition of 5-methylTHF influx requires MTX blood levels in the range of about 5-10 μM, while greater than 90% suppression requires MTX concentrations in excess of 50 μM. Further, differences in the endogenous 5-methylTHF level over a range of 0.1 to 1 μM only negligibly affect the degree of inhibition produced by a given extracellular MTX concentration. Of course, with repeated rescue cycles, much higher levels of THF cofactor might accumulate within the extracellular compartment, sharply reducing the inhibitory effects of the subsequent infusion of MTX on THF cofactor influx into the cell. It should be pointed out, however, that the degree of inhibition of THF-cofactor influx by MTX that is necessary to appreciably retard THF-dependent reactions has not been established, ie, only a small percent of the usual 5-methylTHF flux may meet cellular requirements.

This analysis also suggests that some low-dose regimens for MTX which are clinically effective probably do not produce sufficient extracellular MTX concentrations to appreciably inhibit 5-methylTHF transport into tumor cells. This may represent conditions in which 5-methylTHF is not converted to coenzyme forms required for DNA and RNA synthesis when THF synthesis is blocked.

Another consequence of MTX entry into the cell on the THF-cofactor carrier will be the exit of free and loosely bound THF cofactors from the cell (countertransport). Although MTX would not be expected to accelerate the exit of THF-cofactor forms which do not penetrate the cell membrane (ie, polyglutamates), it would accelerate the exit of cofactor forms to which the cell membrane is permeable as they might be converted from "storage forms." The extent to which this effect is important would depend upon the level of extracellular MTX achieved. Only those MTX levels that would appreciably retard influx (fig 3) would have a comparable inhibitory effect on the free THF-cofactor level within the cell. A countertransport of endogenous THF cofactors from the cell would be of major importance during the early phases of MTX infusion, since as MTX accumulates within the cell, it will compete with intracellular THF cofactors for efflux and eventually reduce the net flow of THF cofactors out of the cell. However, given a sufficient MTX blood level, the cells would be cleared of exchangeable THF cofactors rapidly and the rate of re-entry of THF cofactors into the cell might be too slow to be of much importance. During this phase of the MTX protocol, the presence of a low-affinity transport route for these compounds, while facilitating the transport of MTX into the cell (*see* below), would not influence fluxes of endogenous THF cofactors since their concentrations are so low that they would be transported exclusively by the high-affinity carrier.

Transport Phenomena During the 5-FormylTHF "Rescue"

With respect to transport phenomena, the effectiveness of the rescue will depend upon *(a)* the rate of transport of 5-formylTHF into the cell, and *(b)* the effect of that translocation on the rate at which MTX is transported into and out of the cells as the MTX blood level falls during the "rescue." These factors are considered in the following section.

Effect of the 5-FormylTHF "Rescue" on MTX Transport Into and Out of Cells

Although addition of 5-formylTHF to the extracellular compartment will accelerate net efflux of exchangeable intracellular MTX and reduce the subsequent re-entry of this MTX into the cell during the interval over which the MTX blood level falls following cessation of the MTX infusion, a quantitative consideration suggests that unless the blood levels of 5-formylTHF produced during the "rescue" are quite high, the effects on MTX transport are not important. Figure 4 illustrates the relationship between the percent inhibition of MTX influx by 5-formylTHF during the "rescue" as a function of time following cessation of the MTX infusion, a reflection of the falling MTX blood level. This relationship is considered for four different initial MTX levels (1, 10, 100, and 250 μM) and four different 5-formylTHF blood levels during the "rescue." From this analysis, several observations emerge: (a) The lower the MTX blood level following cessation of the MTX infusion, the more rapid the onset of 5-formylTHF inhibition of MTX influx. *(b)* As the initial MTX blood level is increased, there is an increasing delay in the onset of inhibition of MTX influx for any of the 5-formylTHF blood levels, although ultimately, the degree of inhibition achieved is the same regardless of the initial MTX concentration. *(c)* At 5-formylTHF blood levels achieved in usual rescue protocols (0.5-1 μM) maximum inhibition of MTX influx is small (<20%). Appreciable inhibition of MTX influx requires 5-formylTHF levels in the range of 5-10 μM, but maximum inhibition, even at these levels, is only 50% and 65% respectively. *(d)* Since the effect of 5-formylTHF on the free intracellular MTX level would parallel changes in MTX influx, effects of 5-formylTHF on this component of intracellular MTX would be equally small. Further, if there is a significant transport of MTX by passive diffusion or by a low-affinity carrier at high MTX levels, then the effects of 5-formylTHF on MTX transport during the early phase of the "rescue" (when the MTX influx would be less susceptible to inhibition by 5-formylTHF) would be even smaller.

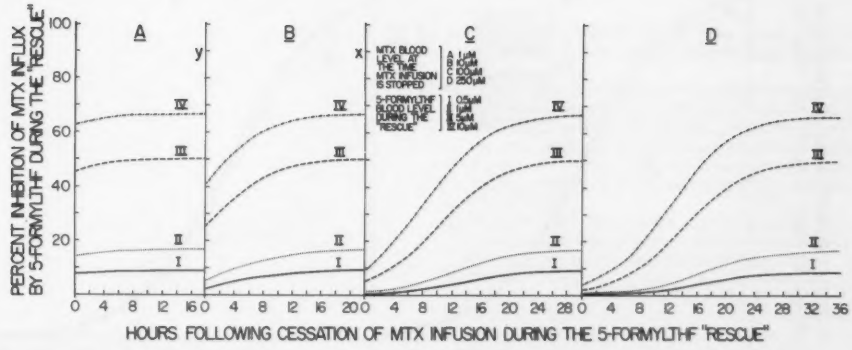
PERCENT INHIBITION OF MTX INFLUX BY 5-FORMYLTHF DURING THE "RESCUE"
A
B
C
D
MTX BLOOD LEVEL AT THE TIME MTX INFUSION IS STOPPED
A 1 μM
B 10 μM
C 100 μM
D 250 μM
5-FORMYLTHF BLOOD LEVEL DURING THE "RESCUE"
I 0.5 μM
II 1 μM
III 5 μM
IV 10 μM
HOURS FOLLOWING CESSATION OF MTX INFUSION DURING THE 5-FORMYLTHF "RESCUE"

FIGURE 4.—Inhibitory effect of 5-formyl THF administered during the "rescue" on the influx of MTX into cells following cessation of the MTX infusion. The rate of decline of the MTX blood level was estimated to be exponential for this analysis with a plasma disappearance half-life of 3 hours. Percent inhibition of MTX influx by 5-formylTHF was computed using a modification of the equation described in the legend to fig 3 with the K_c for MTX and the K_i for 5-formylTHF both estimated at 5 μM. Panels A-D illustrate this relationship at four different initial MTX blood levels. Lines I-IV in each panel refer to this relationship at four different 5-formylTHF levels achieved during the rescue.

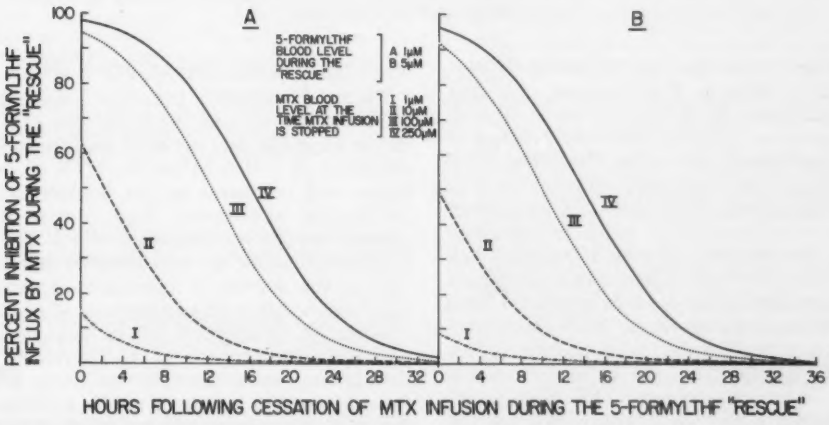
PERCENT INHIBITION OF 5-FORMYLTHF INFLUX BY MTX DURING THE "RESCUE"
A
B
5-FORMYLTHF BLOOD LEVEL DURING THE "RESCUE"
A 1 μM
B 5 μM
MTX BLOOD LEVEL AT THE TIME MTX INFUSION IS STOPPED
I 1 μM
II 10 μM
III 100 μM
IV 250 μM
HOURS FOLLOWING CESSATION OF MTX INFUSION DURING THE 5-FORMYLTHF "RESCUE"

FIGURE 5.—Effect of the falling MTX blood level following cessation of the MTX infusion on the influx of 5-formylTHF into cells during the "rescue." Lines I-IV in each panel indicate this relationship at four different initial MTX blood levels. Panels A and B illustrate this relationship when the 5-formylTHF blood levels achieved are 1 and 5 μM respectively. Estimated constants were the same as described in the legend to fig 4.

Effect of the MTX Blood Level Following Cessation of the MTX Infusion on 5-FormylTHF Delivery into the Cell During the "Rescue"

5-FormylTHF may "rescue" cells by *(a)* bypassing the metabolic block induced by MTX, and *(b)* inducing an efflux of free intracellular MTX, resulting in the dissociation of MTX from low-affinity binding sites and the restoration of THF synthesis (*see* accompanying paper [21]). The data described above would suggest that the effect of 5-formylTHF on the exchangeable intracellular MTX level at usual 5-formylTHF blood levels achieved in the "rescue" is negligible. Hence, the efficacy of the "rescue" of normal host cells would depend upon achieving a rate of 5-formylTHF influx sufficient to sustain essential THF-dependent biosynthetic processes. The extent to which MTX will inhibit 5-formylTHF influx into cells will depend upon the levels of MTX in the blood following cessation of the MTX infusion relative to the concentration of 5-formylTHF achieved during the "rescue," and the affinities of these substances for the high-affinity carrier. Figure 5 describes the relationship between the percent inhibition of 5-formylTHF influx by MTX during the

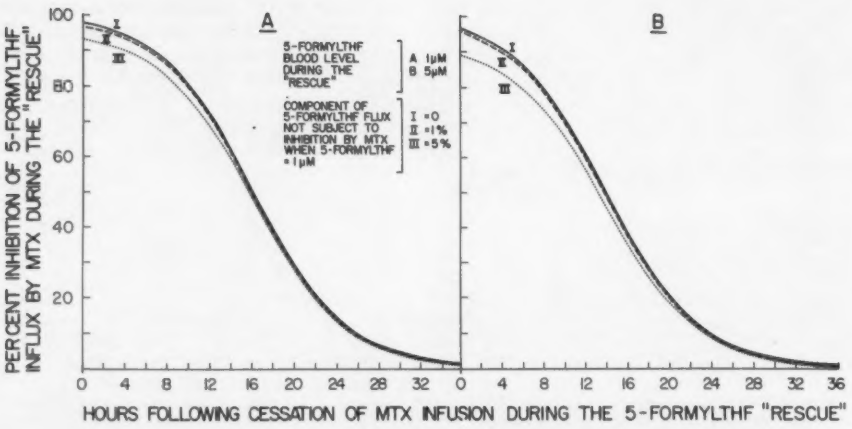

FIGURE 6.—Effect of the falling MTX blood level following cessation of the MTX infusion on the influx of 5-formylTHF during the rescue in the presence and absence of an alternate transport pathway which is uninhibited by MTX. Line I refers to this relationship when the second pathway is absent; lines II and III are when the rate constants for the second pathway are 0.00841 and 0.04385 Min^{-1} respectively, such that when the 5-formylTHF level is 1 μM, the components of the total flux mediated by this route would be 1% and 5% respectively. The two panels reflect this relationship when the 5-formylTHF level is 1 or 5 μM. The kinetic constants utilized in this graph are outlined in the legend to fig 4.

"rescue" as a function of the time following the cessation of the MTX infusion. This analysis considers a broad range of initial MTX blood levels (1, 10, 100, and 250 μM) and two 5-formylTHF blood levels (1 and 5 μM). It can be seen that when the initial MTX blood level is high, there is marked suppression of 5-formylTHF influx so that at 250 μM MTX, more than 90% inhibition of 5-formylTHF influx is sustained for more than 6 hours after the MTX infusion has stopped. This suppression is only slightly decreased when the 5-formylTHF blood level is increased from 1 to 5 μM. However, when the initial MTX blood level is lower, inhibition of 5-formylTHF influx is smaller and falls more rapidly after the cessation of the MTX infusion. When the 5-formylTHF blood level during the "rescue" is increased from 1 to 5 μM, the initial inhibition is reduced and the rate of fall of this inhibition is increased, but these effects are small.

The effects of a second transport route for 5-formylTHF not subject to inhibition by MTX are demonstrated in figure 6. This analysis is based upon a possible second transport component for 5-formylTHF comprising 1% or 5% of the total 5-formylTHF flux when the 5-formylTHF blood level is 1 μM. The initial MTX blood level is 250 μM and the percent inhibition of 5-formylTHF influx as the MTX blood level falls is evaluated at a 5-formylTHF blood level of either 1 or 5 μM. It may be seen that the presence of a second transport route for 5-formylTHF limits the maximum inhibition that can be achieved by MTX, but nonetheless the maximum inhibitory effect remains very high. The effects of such a second component would become increasingly important as 5-formylTHF increases to levels much higher than achieved in usual "rescue" protocols.

Figure 7 indicates the relationship between the MTX blood level and the inhibition of 5-formylTHF influx when the *ratio* of MTX to 5-formylTHF is held constant at 2. Inhibition by MTX of 5-formylTHF influx first increases as the concentration of these substances approaches their carrier K_t's and the system saturates, following which the rise in the inhibitory effect is considerably smaller (line I). Hence, the degree of inhibition depends not only upon the relative concentrations of the transported species but, in addition, on the relationship between their concentrations and the carrier K_t's. Figure 7 (line II) also shows how the inhibitory effect of MTX falls at high concentrations if a component of 5-formylTHF transport is mediated by either passive diffusion or by a low-affinity transport carrier not inhibited by MTX.

These considerations suggest that when the MTX blood level is high, early administration of small doses of 5-formylTHF probably does not influence the effectiveness of the "rescue," while the later administration of this folate cofactor may be the crucial interval over which 5-formylTHF effectively enters the cell to provide THF-cofactor substrate. It is only when the MTX blood level is low that 5-formylTHF, administered immediately after the MTX infusion, will effectively enter the cell. However, since the critical rate of 5-formylTHF influx required to meet cellular demands has not yet been established, even under conditions in which 5-formylTHF influx is reduced by a factor of 80% or 90% over the first few hours of rescue (as with MTX blood levels in excess of 100 μM), the residual

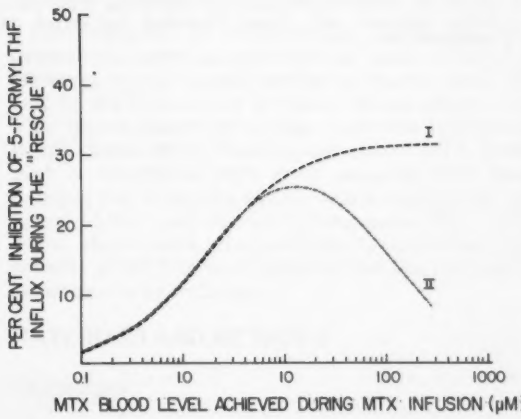

FIGURE 7.—Initial inhibition of 5-formylTHF influx during the rescue when the ratio of the initial MTX level to 5-formylTHF is 2 under conditions in which transport is by the high-affinity carrier alone (I) or the high-affinity carrier and an alternate transport route for 5-formylTHF not inhibited by MTX (II). The magnitude of the rate constant for the second component for 5-formylTHF transport is 0.02525 min^{-1}, a level at which 1% of the total flux for 5 μM 5-methylTHF would be mediated by this route. The other kinetic constants employed are described in the legend to fig 4.

10%-20% of 5-formylTHF penetrating the cell may be sufficient to meet cellular THF cofactor demands.

RELEVANCE OF PHARMACOKINETIC DATA IN ANIMAL TUMOR CELLS TO "RESCUE" PROTOCOLS IN MAN

The characteristics of the membrane transport of MTX and THF cofactors as related to possible interactions during rescue protocols described in figures 3-7 are derived from estimates of transport constants and other experimental data relevant to a variety of animal tumor cells. This may not, however, adequately reflect transport parameters in normal and malignant human cells, although studies in some human tissues suggest that there is considerable similarity (6,13,30,31). These considerations are presented in an attempt to demonstrate that the conceptualization of the interaction between MTX and THF cofactors at the level of the cell membrane may provide insight into the design of effective rescue protocols. It is hoped that this approach may stimulate the investigation of transport and cellular pharmacokinetic parameters (21) in normal susceptible host tissues as well as in the variety of solid tumors that are now being treated with rescue protocols.

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Membrane Transport of Methotrexate (NSC-740) in Human Neoplastic Cells[1]

Richard A. Bender[2,3,4]

The transport of methotrexate (MTX) has been extensively investigated in animal tumor cells (1-10). However, only a limited number of studies are available on MTX transport in human tumor cells (11-16). This report describes studies from this laboratory which characterize various aspects of MTX transport in neoplastic cells from patients with acute lymphocytic leukemia (ALL), acute myelocytic leukemia (AML), and Burkitt's lymphoma (BL). Additional studies are presented which relate the cytotoxicity of MTX to both intracellular and extracellular drug concentrations.

MATERIALS AND METHODS

Chemicals

[3',5'-^{3}H]-MTX was obtained from Amersham/Searle Corporation, Des Plaines, Illinois, or Dhom, North Hollywood, California. Purification was carried out as previously described (1) using linear gradient elution with ammonium bicarbonate buffer. [6-^{3}H]-deoxyuridine was obtained from Amersham/Searle Corporation. Sodium azide was obtained from Fisher Scientific, Silver Spring, Maryland. Sodium iodoacetate was obtained from Eastman Kodak Company, Rochester, New York. *p*-Chloromercuribenzoic acid was obtained from Schwarz/Mann, Orangeburg, New York. Folic acid was obtained from Calbiochem, Los Angeles, California. Sodium cephalothin, hydrocortisone sodium succinate, and vincristine[5] were the commercially available clinical preparations.

Cells and Media

Tumor cells were obtained from patients (table 1) whose peripheral blood contained greater than 90% blast cells. None of the patients had received any chemotherapy within 2 weeks of study nor had any patient received MTX within 2 months of study. Details of cell preparation, incubation, and analytic techniques have been previously described (1,16). Cells were suspended in Eagle's minimal essential media at 37°C without added serum or folic acid and with pH maintained at 7.4 in the presence of 95% O_2-5% CO_2, unless otherwise indicated. Significant metabolism of MTX did not occur during the experimental period (16).

Cytotoxicity studies were performed on leukemic or lymphomatous cells obtained from the bone marrow of patients resistant to drug combinations containing MTX. Neoplastic cells were separated from normal marrow elements by isolation on a Ficoll column as described by Evans et al (17). Cells were suspended in solutions of purified unlabeled MTX at various concentrations for 120 minutes prior to the addition of tritiated deoxyuridine for an additional 30 minutes. Deoxyuridine incorporation into trichloroacetic acid-precipitable material was then expressed as the percent of incorporation in control samples containing no MTX.

MTX TRANSPORT IN TUMOR CELLS

Carrier-Mediated Uptake

The transport of sugars and amino acids in mammalian cells and bacteria has been carefully studied

TABLE 1.—Patient information

Patient No.	Cell type	Previous therapy*
1	AML	None
2	AML	ARA-C/6-TG, DNR, MTX, GNZ
3	AML	ARA-C/6-TG, DNR, methyl-GAG
4	AML	ARA-C/6-TG, DNR
5	AML	None
6	AML	None
7	AML	ARA-C/6-TG, DNR
8	ALL	POMP, VDP, ASP
9	ALL	None
10	ALL	POMP, VDP, ASP
11	ALL	None
12	BL	None
13	BL	CYC, VLB, BLEO, PRED
14	BL	None
15	BL	CYC

*ARA-C = cytosine arabinoside; 6-TG = 6-thioguanine; DNR = daunorubicin; GNZ = guanazole; POMP = prednisone, vincristine, methotrexate, and 6-mercaptopurine; VDP = vincristine, daunorubicin, and prednisone; ASP = L-asparaginase; CYC = cyclophosphamide; BLEO = bleomycin; VLB = vinblastine; and PRED = prednisone.

[1]Methotrexate: CAS reg. No. 59-05-2; glutamic acid, *N*-[*p*-[[(2,4-diamino-6-pteridinyl)methyl]methylamino]benzoyl]-; MTX.

[2]Laboratory of Chemical Pharmacology, Experimental Therapeutics, and Medicine Branch, Medical Oncology, Division of Cancer Treatment (DCT), National Cancer Institute (NCI), Bethesda, Md.

[3]I would like to acknowledge the assistance of Drs. Paul Chang, Brigid G. Leventhal, and John L. Ziegler in obtaining patient material. In addition, the helpful discussions with Drs. I. David Goldman, Robert L. Dedrick, and Vincent T. Oliverio are acknowledged.

[4]*Reprint requests to:* Richard A. Bender, MD, Medical Oncology, DCT, NCI, Bldg 10, Rm 12N226, National Institutes of Health, Bethesda, Md 20014.

[5]Vincristine: NSC-67574; CAS reg. No. 2068-78-2; leurocristine, sulfate (1:1).

and was found to be compatible with a carrier-mediated or -facilitated process (18). The experimental evidence to be presented suggests that a similar process is responsible for the membrane transport of MTX in human neoplastic cells. This process is schematically represented in figure 1 and is similar to that already described in L1210 leukemia cells by Goldman et al (1). In the human model, extracellular MTX enters the cell by traversing the cell membrane associated with a carrier from which it dissociates upon reaching the intracellular milieu. Because of the extremely high affinity for MTX of dihydrofolate reductase (DHFR), the target enzyme of MTX, all drug entering the cell is bound by DHFR until its binding sites are saturated. The drug moves only unidirectionally into the cell during this period and its rate of movement is referred to as the influx velocity (V_i). Its rate of entry is dependent on both the extracellular drug concentration and the membrane permeability of the cell.

Following saturation of high-affinity sites, intracellular drug continues to accumulate at a rate dependent on the separate rates of influx and efflux. When these rates are equivalent, the concentration of exchangeable intracellular drug becomes constant and a "steady state" is reached.

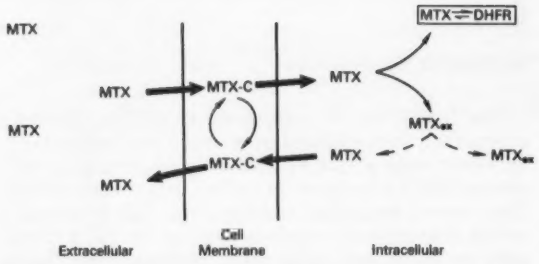

FIGURE 1.—Schematic representation of carrier transport model for MTX. MTX enters the cell in association with a carrier (C) and can then bind tightly to DHFR or remain exchangeable (MTX_{ex}). Exchangeable drug can remain intracellular or efflux via a carrier.

MTX uptake has been studied in human ALL, AML, and BL cells. The time course of their drug uptake is seen in figure 2. In the presence of a constant extracellular concentration, MTX uptake proceeds rapidly over 80 minutes following which the rate of accumulation slows, but only in the AML

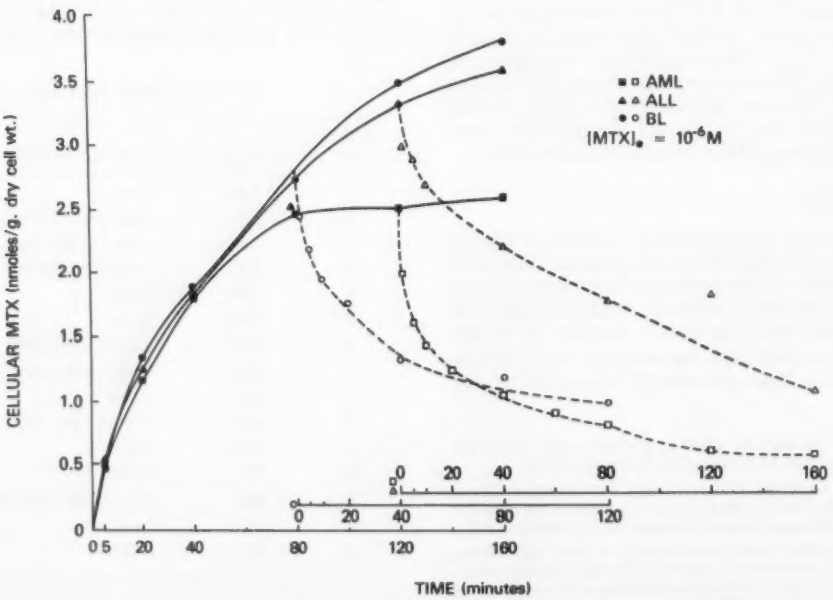

FIGURE 2.—Uptake and efflux of MTX in human neoplastic cells. Each point represents the mean value of duplicate determinations in each patient in table 1. Uptake curves are represented by a solid line and a solid symbol and efflux curves by an interrupted line and an open symbol. The time course for uptake is on the abscissa with that for efflux represented by separate numbered lines above the abscissa. The symbol at the beginning of each of these lines designates the cell type to which it corresponds.

TABLE 2.—Effect of PCMB, folic acid, and folinic acid on MTX uptake in human neoplastic cells

Drug	Concentration (M)	Uptake time (min)	V_i (at 5 mins) or uptake value (at 160 mins) in—* AML	ALL	BL
PCMB	2×10^{-3}	5	15	24	20
		160	56	73	65
Folic acid	10^{-4}	5	50	58	65
		160	69	78	80
Folinic acid	10^{-6}	5	64	67	60
		160	75	72	73

*Each number represents the mean percent of control for duplicate determinations in 3 patients with each cell type. Uptake experiments were carried out as described at 37°C with extracellular MTX concentrations of 10^{-6}M. The early (5-min) time point was chosen to show the reduction of V_i caused by these agents. The later time point demonstrates that control levels of drug were never attained in the presence of these agents.

cells does this rate approach equilibrium. The ALL and BL cells continue to accumulate drug, albeit at a slower rate. Accordingly, a steady state was never observed in ALL and BL cells under the conditions of these investigations. Comparison of the mean 160-minute uptakes among the three cell types using paired-t-analysis failed to reveal any significant differences in total or exchangeable intracellular MTX. Further, a similar comparison between previously treated or untreated patients of each cell type revealed no significant differences. Specific analysis of patients previously treated with MTX was prevented by small patient numbers.

Compounds structurally related to the antifolate MTX are known to inhibit MTX influx in animal tumors (3). Similar results were obtained when folic acid and folinic acid (citrovorum factor [CF])[6] were studied in human neoplastic cells (table 2). Cells were suspended in 10^{-6} M MTX solutions to which folic acid at 10^{-4} and 10^{-6} M and CF at 10^{-6} M were added. Folic acid at 10^{-6} M did not affect either the V_i or the 160-minute uptake value. However, folic acid at 10^{-4} M and CF at 10^{-6} M significantly reduced the V_i (5-minute points) by 42% and 36% respectively, and the 160-minute uptake value was reduced by 24% and 27% respectively. There was no significant difference between the magnitude of the inhibition noted among the three cell types studied. The nature of the inhibition was specifically explored in ALL cells by a Lineweaver-Burk analysis of the CF effect (fig 3) where the common y-intercept indicates that this inhibition is competitive in nature. Although not specifically studied, the mechanism for the folic acid effect may be similar to that for CF. However, the

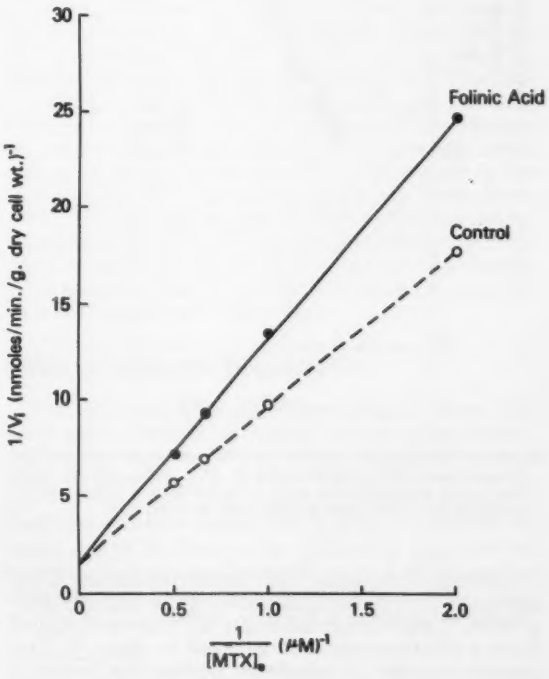

FIGURE 3.—Double reciprocal plot of the effect of 10^{-6} M folinic acid on the influx kinetics of MTX uptake in ALL cells. Each point represents the mean value for $\frac{1}{\text{uptake velocity}}$ in three patients.

[6]Citrovorum factor: NSC-3590; CAS reg. No. 6035-86-5; glutamic acid, *N*-[p-[[(2-amino-5-formyl-5,6,7,8-tetrahydro-4-hydroxy-6-pteridinyl) methyl]amino]benzoyl]-, calcium salt.

necessity for very high levels of folic acid relative to MTX in order to inhibit MTX uptake suggests a low affinity between folic acid and the MTX carrier site. It is also possible that several carriers are responsible for the transmembrane movement of the various folates in man and that MTX and folic acid do not share the same carrier mechanism.

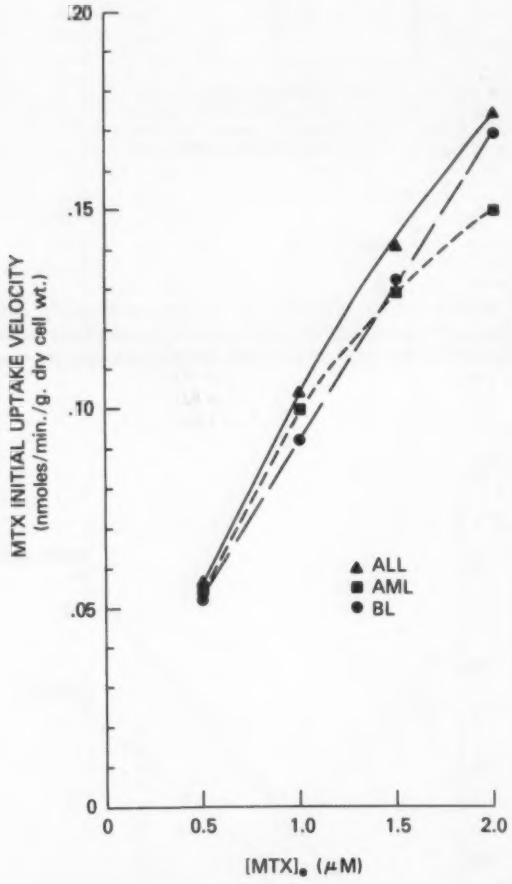

FIGURE 4.—Relationship between the MTX uptake velocity and the extracellular MTX concentration in ALL, AML, and BL cells. Each point represents the mean value for uptake velocity as determined in at least three patients with each cell type.

Additional evidence for carrier-mediated MTX uptake is the relationship between the unidirectional MTX V_i and the extracellular MTX concentration. Such a relationship is illustrated in figure 4. The uptake velocity is calculated during the initial 5 minutes of MTX uptake, since uptake in excess of this interval leads to accumulation of exchangeable intracellular drug and efflux, then, becomes a consideration. Only a narrow range of extracellular concentrations (0.5-2.0 μM) have been studied thus far, and so saturation of uptake has not been observed. However, attention to the ALL and particularly the AML curve reveals a decrease in slope with increasing MTX concentration. This suggests that eventual saturation will occur allowing calculation of a maximal unidirectional influx velocity (V_{max}) and also a Michaelis constant (K_m) equal to the substrate concentration at 1/2 V_{max}. These values can be calculated from the y-intercept of the control line (ALL) in figure 3. The reciprocal of the y-intercept corresponds to the V_i at infinitely high MTX concentrations (V_{max}) and is equal to 0.71 nmols/min/g dry cell weight. By substituting this value for V_{max} in equation (1) and using the value for V_i at 10^{-6} M in ALL cells obtained from figure 4, the K_m can be calculated. Its value is 5.82×10^{-6} M.

$$V_i = \frac{V_{max}\ C}{K_m + C} \qquad (1)$$

$$\text{thus, } K_m = \frac{V_{max}\ C}{V_i} - C$$

In addition to the inhibitory effect of folates on MTX uptake, anions inhibit MTX influx and reduce the steady-state distribution ratio (3), the ratio of the intracellular concentration of exchangeable MTX divided by the extracellular MTX concentration. It has been suggested that anions also compete with MTX for carrier binding sites to inhibit uptake. The anions that have been studied in man are hydrocortisone sodium succinate (HC) and sodium cephalothin (KEF) as they exist at physiologic pH. Both of these compounds inhibit the influx of MTX and diminish the steady-state distribution ratios in AML cells. HC and KEF significantly reduce the distribution ratios by 28% and 20% respectively at concentrations of 10^{-4} and 5×10^{-5} M (fig 5). Similar inhibition of both MTX influx and 160-minute MTX distribution ratios is seen in ALL and BL cells when incubated with HC or KEF at the indicated concentrations, which are estimates of the peak levels achieved in man following a single standard intravenous (iv) dose. The precise mechanism for the anion effect remains unclear.

Further evidence for carrier mediation is the inhibition of uptake in all three cell types caused by exposure to *p*-chloromercuribenzoic acid (PCMB), a potent sulfhydryl inhibitor. PCMB significantly inhibits MTX uptake and reduces the steady-state distribution ratios to 56%, 73%, and 65% of control for AML, ALL, and BL cells respectively (table 2). These effects are similar to those observed in L1210 murine leukemia and have been interpreted to suggest that the integrity of the active carrier site involves sulfhydryl groups (1). Such an interpretation is consistent with the observed PCMB effect in human neoplastic cells.

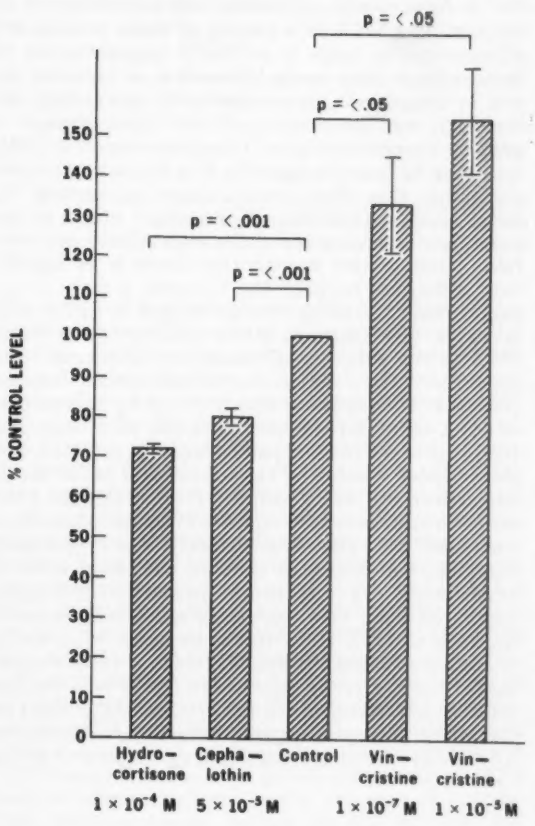

FIGURE 5.—Inhibition and enhancement of MTX uptake by chemotherapeutic agents in AML cells in vitro. The shaded area represents the mean percent ± SEM of the control level of MTX taken up by AML cells in the presence of each "drug" at the 160-min or steady-state point. *P* values are determined by paired-t-test analysis. Extracellular MTX concentration = 10^{-6} M (from ref 16).

Thus, carrier mediation of MTX uptake in human neoplastic cells is supported by two observations: (*a*) the suggestion of saturation kinetics with competitive inhibition by folinic acid, and (*b*) the inhibitory effect of the sulfhydryl inhibitor, PCMB, on MTX uptake. It is important to recognize that these observations have been made at extracellular MTX concentrations of about 10^{-6} M. At higher drug levels a deviation from expected kinetics may occur. Recent studies by Goldenberg et al (20) and Lyons et al (21) on cyclophosphamide and mechlorethamine transport respectively in leukemic cells suggest that two overlapping mechanisms for drug uptake may exist. Firstly, there is a low concentration mechanism which is dominant at 10^{-4} M or less; it is characterized by high-affinity, low-capacity uptake and is consistent with a carrier-mediated process. Secondly, there is a high concentration mechanism which dominates at 10^{-3} M or greater; it is characterized by low-affinity, high-capacity uptake and is consistent with either simple diffusion or a technically nonsaturable transport system. Whereas, the K_m for MTX uptake in man appears to be on the order of 10^{-6} M, the high-affinity, low-capacity system must exist in this range of MTX concentrations. However, recent interest in high-dose MTX infusions, where plasma concentrations of 10^{-3} to 10^{-4} M (22,23) are attained, may provide MTX levels sufficient to "overload" the low-capacity carrier and allow drug to enter the cell by an alternate route. Such a route could be simple diffusion or a low-affinity, high-capacity transport mechanism. This area is the subject of current investigation.

Cellular Efflux of MTX

In a manner similar to the influx or entry of MTX into the cell, drug can also efflux or exit the cell following saturation of high-affinity intracellular binding sites. This process is also likely to be carrier mediated and energy dependent as will be discussed in the next section. The shape of the efflux curves for AML, ALL, and BL cells suggests a biphasic process (fig 2). There is an initial rapid loss of intracellular drug over the first 20 minutes followed by a slower attrition process which terminates when all exchangeable drug has left the cell leaving only the non-exchangeable tightly bound fraction. The continued slow efflux of MTX from ALL and BL cells may reflect diminishing cell viability with increasing incubation time. Examination of the terminal efflux values in figure 2 suggests some differences in the intracellular MTX levels among the cell types. However, paired-t-test analysis of these mean values indicates no statistically significant differences. Further, comparing the mean values for previously treated and untreated patients in each cell type revealed no significant differences.

Effect of Metabolic Inhibitors

Previous studies (11) in human leukemic blast cells have shown that MTX uptake is nonconcentrative, indicating that the steady-state distribution ratio does not exceed unity. Similar studies on AML, ALL, and BL blast cells also suggest that MTX uptake is nonconcentrative under the in vitro conditions of study (table 3). Since only AML cells appeared to reach a steady state during the period of incubation (160 minutes), it may be expected that further incubation of ALL and BL cells would produce additional

[7]Cyclophosphamide: NSC-26271; CAS reg. No. 6055-19-2; 2*H*-1,-3,2-oxazaphosphorine, 2-[bis(2-chloroethyl)amino]tetrahydro, 2-oxide, monohydrate.

Mechlorethamine: NSC-762; CAS reg. No. 55-86-7; diethylamine, 2,2'-dichloro-*N*-methyl-, hydrochloride; nitrogen mustard.

accumulation of exchangeable intracellular MTX thereby raising the distribution ratio above the listed value. Inspection of their uptake curves allows estimation of a steady-state value in the range of 4.0-4.5 nmols/g dry cell weight. If the values of non-effluxable drug remain unchanged, then the distribution ratios remain less than unity. However, since MTX is a charged moiety at physiologic pH, the distribution ratio of MTX need not exceed unity for transport to be "uphill" or energy dependent. The resolution of this problem requires knowledge of the transmembrane potential in AML, ALL, and BL cells in order to determine if MTX is being accumulated against an electrochemical potential.

An indirect way of examining the question of energy dependence is to expose cells to metabolic poisons to determine their effect on MTX uptake. If energy coupling is essential for drug influx, then metabolic poisons should diminish the intracellular drug levels. However, only lowering the bath temperature had this effect. Exposure to solutions of azide and iodoacetate, inhibitors of both anaerobic and aerobic glycolysis respectively, and to solutions of vincristine, a drug reported to inhibit energy coupling to the membrane "pump" (7), augmented the intracellular drug levels.

A summary of the results of 4°C incubations on MTX uptake is found in table 4. An 80%-89% reduction in the initial uptake velocity (note 5-minute values) and a 67%-75% reduction in the 160-minute distribution ratios were observed in the three cell types. However, the magnitude of this effect is far less in human cells where an approximate Q_{30} of 3-4 is observed, whereas a Q_{10} of 7 is noted in L1210 cells.

Exposure of neoplastic cells to 10^{-2} M azide and 10^{-3} M iodoacetate augmented the accumulation of intracellular MTX. The results of these studies are summarized in table 5. An early augmentation of intracellular drug levels followed by a terminal decay in drug levels, associated with decreasing cell viability, was observed in all cell types studied. A graphic representation of this phenomenon in AML cells may be found in figure 6. It is attractive to conclude that this effect results from augmenting V_i, but a close examination of the initial phase of uptake prior to saturation of the high-affinity intracellular binding sites reveals that there is no significant difference between the V_i under control or experimental conditions. Similar studies in L1210 cells reveal a 15% increase in the unidirectional V_i of MTX in the presence of azide and iodoacetate (2). However, the major azide/iodoacetate-induced change in both systems was reflected by an analysis of MTX efflux from cells preloaded with drug and then suspended in an azide/iodoacetate solution. For this purpose, the initial rate of efflux of MTX (determined over the first 5 minutes) from preloaded AML cells in the presence or absence of azide/iodoacetate was examined. The efflux velocity was 0.18 nmols/min/g dry cell weight in controls compared to 0.068 nmols/min/g dry cell weight in azide/iodoacetate-incubated cells. This represents a 260% decrease in the rate of MTX efflux in the presence of azide/iodoacetate in human neoplastic cells, a value comparable to that already reported in L1210 cells (3). The relative selectivity of the azide/iodoacetate effect on the efflux process suggests different requirements for energy coupling between drug influx and efflux.

TABLE 3.—MTX distribution ratios in human neoplastic cells*

Cell type	Drug uptake at 160 mins (A)	Non-effluxable drug (B)	A − B	$\frac{[MTX]_i}{[MTX]_e}$
AML	2.60	0.58	2.02	0.54
ALL	3.60	1.10	2.50	0.67
BL	3.80	1.00	2.80	0.75

*Exchangeable intracellular drug concentrations ($[MTX]_i$) were calculated using the dry weight/intracellular volume ratios of Goldman et al (1). The values in columns A and B are derived from fig 2. Extracellular MTX concentration = 10^{-6} M.

TABLE 4.—Effect of 4°C incubation on MTX uptake in human neoplastic cells

Cell type	MTX uptake at—* (mins)					
	5	20	40	80	120	160
AML	11	14	16	20	25	26
ALL	18	19	22	24	25	25
BL	20	26	28	27	33	—

*Each number represents the mean percent of control (37°C uptake) for duplicate determinations in 3 patients with each cell type. Uptake experiments were carried out as described at 4°C with extracellular MTX concentrations of 10^{-6} M.

TABLE 5.—Effect of sodium azide/sodium iodoacetate on MTX uptake in human neoplastic cells

Cell type	Azide/iodoacetate uptake at—* (mins)					
	5	20	40	80	120	160
AML	154	178	206	230	172	89
ALL	100	178	204	186	104	67
BL	137	154	123	105	—	82

*Each number represents the mean percent of control for duplicate determinations in 3 patients with each cell type. Uptake experiments were carried out as described at 37°C with extracellular MTX concentrations of 10^{-6} M. Concentrations of azide and iodoacetate were 10^{-2} and 10^{-3} M respectively.

This notion is further supported by the effect of vincristine on the MTX uptake of neoplastic cells. Vincristine significantly augments the steady-state values for intracellular MTX by 33% and 54% respectively at concentrations of 10^{-7} and 10^{-5} M in AML cells (fig 5). Similar enhancement is also seen in ALL and BL cells. A plasma concentration of 10^{-7} M is easily reached following iv administration of a 2-mg dose, but 10^{-5} M represents a pharmacologic level used only for experimental purposes. The mechanism by which vincristine produces this enhancement of intracellular drug levels was examined by suspending two identical populations of AML cells in 10^{-6} M MTX solutions to which vincristine at 10^{-5} M (vincristine curve) or no vincristine (control curve) had been added (fig 7). Following 120 minutes of uptake, cells were washed and resuspended in vincristine or control solutions without MTX. A detailed analysis of the initial uptake phase of the vincristine and control curves (0-5 minutes) revealed no significant differences. However, the initial efflux rate of MTX was significantly slower in the presence of vincristine. With continued incubation, the intracellular level of MTX in the vincristine-incubated cells tended to approach that of control, but due to decreased cell viability with continued incubation, an equivalence point was never reached. However, if vincristine-incubated cells were washed free of vincristine and resuspended in vincristine-free media, rapid efflux of MTX occurred so that a control level was reached by 80-100 minutes. These observations indicate that vincristine enhances intracellular MTX level by retarding efflux without affecting influx. Further, high-affinity binding to intracellular sites is unaffected by vincristine. Thus, vincristine provides a useful method for increasing exchangeable intracellular MTX at external MTX concentrations of 10^{-6} M. Whether or not vincristine is similarly useful at significantly higher MTX concentrations is uncertain.

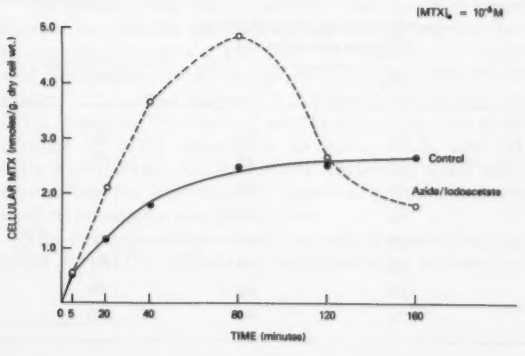

FIGURE 6.—Effect of azide and iodoacetate (10^{-2} and 10^{-3} M respectively) on MTX uptake in AML cells. Each point represents the mean value for uptake as determined in three patients.

The ability of metabolic poisons to differentially influence the rates of MTX influx and efflux is curious. It suggests different sensitivities and energy sources for these processes and further suggests that uptake may, in fact, be energy dependent while being nonconcentrative. If this is the case, a complex process exists whereby the cellular electrochemical potential is determined by a balance of two energy dependent processes. The first process is responsible for driving MTX uphill into the cell against an electrochemical potential. This pathway is not immediately affected by metabolic poisons by virtue of using energy reserves or an alternate energy pathway. The second process requires energy to sustain transport of MTX out of the cell. This process relies on endogenous energy metabolism and, hence, is easily affected by metabolic poisons. Such a mechanism has already been postulated for MTX transport in L1210 murine leukemia cells (1,3) and may also be relevant to its transport in human neoplastic cells. If MTX transport occurs in this manner, then the cellular milieu becomes important as a determinant of drug uptake.

CYTOTOXICITY OF MTX

The principal rationale for high-dose MTX is that increasing the extracellular drug levels produces a greater cytocidal effect than is seen at lower doses. The ability of such "high" doses to induce responses in previously unresponsive or poorly responsive solid tumors (24-26) has been demonstrated. However, it does not follow from these observations that indefinite increases in extracellular MTX concentrations will give improved tumor responses. Based on what

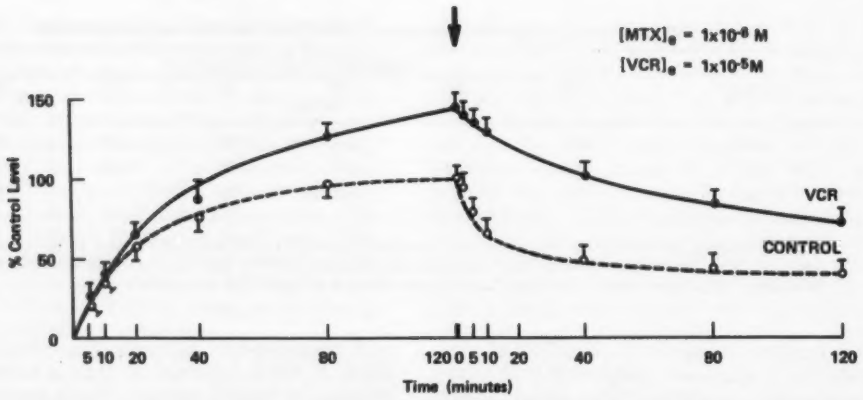

FIGURE 7.—MTX uptake and efflux in the presence and absence of vincristine. AML cells were incubated in the presence of MTX and vincristine (vincristine curve) or MTX alone (control curve) at 37°C. After 120 mins (bold arrow), cells were washed twice and resuspended in medium with or without vincristine respectively, and the efflux curves were plotted. Each point represents the mean ± SEM of three experiments (from ref 16).

currently known about the mechanism of action of MTX, a maximal effect requires saturation of all DHFR binding sites and, presumably, some exchangeable intracellular MTX as well. It has been demonstrated that increasing intracellular MTX in excess of the tightly bound fraction enhances the therapeutic efficacy of MTX in L-cell mouse fibroblasts (27) and in Ehrlich ascites tumor cells (28) in vitro. It appears that this component of intracellular MTX is necessary for effective inhibition of DNA, RNA, and protein synthesis in these cell types. Further, studies by Zager et al (6) clearly indicate that the survival of mice is enhanced by pretreating animals with vincristine prior to administering MTX. These studies suggest that exchangeable intracellular drug is also important for in vivo cytotoxicity. Recent data from Sirotnak and Donsbach (29) may suggest an alternate explanation, but these studies require further confirmation.

Examination of figure 2 reveals that a 120-minute exposure to 10^{-6} M MTX solutions is sufficient to saturate all high-affinity intracellular binding sites, including DHFR, in human ALL, AML, and BL cells in vitro. One could then postulate that 10^{-6} M MTX extracellular levels are sufficient to maximally inhibit DNA synthesis in AML, ALL, and BL cells. To examine this question, bone marrow was obtained from eight patients (four with ALL, three with BL, and one with AML) unresponsive to drug combinations containing MTX and who had not received

TABLE 6.—Deoxyuridine incorporation at various MTX concentrations in human neoplastic cells

Patient No.	Cell type	Clinical response†	Deoxyuridine incorporation‡ at MTX concentrations of—			
			10^{-5} M	10^{-6} M	10^{-7} M	10^{-8} M
1	AML	NR	45	44	54	81
2	ALL	CR	15	17	42	77
3	ALL	NR	59	68	98	—
4	ALL	NR	37	43	74	—
5	ALL	NR	47	49	93	—
6	BL	PR	18	18	63	88
7	BL	PR	15	18	64	100
8	BL	NR	41	74	84	100

*Technical and procedural details are contained in the text.
†CR = complete response; PR = partial response; NR = no response.
‡Expressed as percent of control where controls represent neoplastic cells incubated in the absence of MTX.

MTX within 4 weeks prior to study. Transport studies were performed to assure saturation of high-affinity binding sites, and then deoxyuridine incorporation into trichloroacetic acid-precipitable material was measured. High-affinity sites were saturated during the experimental period (120 minutes) at MTX concentrations of 10^{-7} M or greater, but appreciable levels of exchangeable intracellular drug did not accumulate until 10^{-6} M extracellular levels were reached. The deoxyuridine incorporation data for each patient are summarized in table 6. Deoxyuridine incorporation decreased as extracellular MTX was increased from 10^{-8} to 10^{-6} M. Increasing extracellular MTX from 10^{-6} to 10^{-5} M produced no further significant inhibition of deoxyuridine incorporation, except in patient No. 8. Further, when these patients were subsequently treated with a 24-hour infusion of MTX at a measured plasma concentration of $1\text{-}2 \times 10^{-6}$ M followed by a 12-hour infusion of cytosine arabinoside[8] at 30 mg/m^2, only those patients with in vitro deoxyuridine incorporation of less than 20% of control showed any clinical response. Further, responses did not occur in those patients (Nos. 1, 3, 4, and 5) who failed to respond to 10^{-6} M MTX infusions and in whom the plasma levels were escalated to 5×10^{-6} or 10^{-5} M on subsequent infusions.

These observations suggest that: (*a*) exchangeable intracellular MTX may be required for maximum cytotoxicity, and (*b*) an optimal MTX concentration may exist for each responsive neoplasm above which further escalation produces no additional tumor response. Previous clinical (11,30) and experimental (8) observations support the concept of individual tumor sensitivities to MTX. In order to determine the optimal MTX concentrations, each tumor must be studied individually. Further, only by studying large numbers of patients with each type of responsive neoplasm will a meaningful value for the optimal concentration be obtained.

CONCLUSIONS

The similarities between MTX transport in those human neoplasms studied and in L1210 murine leukemia may suggest a common mechanism for folate and antifolate transport in mammalian cells. More important, it may suggest a common mechanism for MTX transport in human neoplastic cells. The similarities of MTX transport in AML, ALL, and BL cells would support this notion. However, until additional tumors are studied, especially solid tumors, such conclusions are premature.

The carrier-mediated and energy-coupled mechanism for MTX uptake at extracellular concentrations of 10^{-6} M has been discussed. However, because the current use of MTX includes high-dose treatment, a further consideration of possible mechanisms for transport at these plasma levels seems appropriate. At high extracellular MTX concentrations, drug transport may occur by the previously described route or it may occur by simple diffusion or a low-affinity, high-capacity carrier as has been suggested by other investigators (20,21) for cyclophosphamide and mechlorethamine transport at high concentration. Such an alternate route would be advantageous in the treatment of tumors with high optimal concentrations of intracellular MTX or in poorly permeable tumors that were unable to transport lethal amounts of drug at lower concentrations. Further, high MTX concentrations would favor drug permeation of bulky poorly vascularized solid tumor masses whatever the mechanism for transport at high-dose levels. In addition, alternate routes for MTX uptake would minimize or negate the potential advantages of vincristine and the potential disadvantages of anionic substances, such as antibiotics, on drug transport by reducing their effect on intracellular drug levels. However, it is unlikely that the kinetics of CF "rescue" would be affected, as MTX and CF compete for membrane carrier sites in order to enter the cell. Unless the extracellular CF concentration was escalated concomitantly with the MTX concentration, the MTX would tie up all the carrier sites until its concentration declined to the CF concentration, at which time CF could enter the cell competitively and "rescue" could be effected. Since conventional CF doses can be calculated to produce plasma levels of 10^{-6} to 10^{-7} M, a delay in "rescue" of 18 or more hours might be expected following a 6-hour infusion of MTX at 100 mg/kg until the plasma MTX concentration declines to about 10^{-6} M (23). Thus, a 6-hour infusion at these MTX doses may be cytotoxic for 18 or more hours before "rescue" is achieved. Since toxicity of prolonged MTX infusions has been well documented (31,32), the delay in "rescue" of normal tissues becomes an important consideration as MTX doses are increased and infusion times prolonged. If higher MTX doses and longer infusions are found desirable, then CF doses will also need to be increased so that "rescue" can be achieved without delay.

Thus, MTX transport in human neoplastic cells is a complex process whose maintenance is essential for drug entry and the resultant cytotoxicity. The effect of various agents on transport has been examined as well as the possibility of differing effects if alternate transport routes exist at higher extracellular concentrations. The concept of varying optimal extracellular concentrations for different tumors as a function of their individual sensitivities has also been examined. Lastly, the role of exchangeable intracellular MTX and its implications for the mechanism of drug action have been discussed and data have been presented which support this notion. Continued investigation will, it is hoped, clarify the remaining questions.

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[8]Cytosine arabinoside: NSC-63878; CAS reg. No. 69-74-9; cytosine, 1-β-D-arabinofuranosyl-, monohydrochloride.

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CANCER CHEMOTHERAPY REPORTS Part 3 Vol. 5, No. 1 July 1975 Pages 1-62